

THE RESPONSES OF THE SAW-TOOTHED GRAIN BEETLE Oryzaephilus surinamensis
TO PHEROMONES AND FOOD ODOURS.

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Thesis submitted for the degree of Doctor of Philosophy.

Hilary Term 1987.

To Helen

ACKNOWLEDGEMENTS.

This work was conducted as part of the joint Oxford University / M.A.F.F. Slough Laboratory project on "The responses of stored-product insects to pheromones and food attractants."

I am indebted to my supervisor, Dr. Stephen Simpson, for his enthusiasm, advice, assistance and all-enduring optimism. I should also like to thank Dr. Martin Birch for the original conception of the project, and for his interest, help and lab space, and Professor Sir Richard Southwood for his personal support from the outset of this work.

At M.A.F.F., Slough Laboratory, I should like to acknowledge the considerable contribution of Dr. John Chambers, who conducted the insect aerations, produced the carob distillate and taught me how not to misuse the GLC, and David Pinniger, for much encouragement and useful discussion. Dr. A.M. Pierce, Simon Fraser University, British Columbia, Canada, and Prof. K. Mori, Dept. of Agricultural Chemistry, Tokyo kindly supplied samples of synthetic pheromones.

Many thanks are also due to Barbara Luke, for teaching me electron microscopy, Dr. Lester Wadhams, Rothamsted Experimental Station, for helping me with single cell recordings, Professor J.S. Kennedy for his insight and enlightening discussions, and Dr. H.C. Dawkins for statistical advice and computer programmes.

I should also like to express my gratitude to my parents and to Ruth and Geoff for their continued interest and support, and to my late (Great) Uncle Harry for a generous contribution towards my fees. Last, but by no means least, I want to thank my wife Helen, who has had to suffer every phase of this project, yet has kept us both smiling throughout.

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ABSTRACT

The responses of the saw-toothed grain beetle, Oryzaephilus
surinamensis, to food odours and pheromones were investigated at both sensory and behavioural levels to give an integrated picture of olfaction, and the importance of odours in this species.

The sensory capability of the antenna was assessed by a study of the structure, distribution and function of sensilla using SEM and TEM. Seven different sensilla types, including 3 with an olfactory function, were identified, although the total number of olfactory sensilla was low compared with those for mechanoreception.

Antennal responses to odours were studied using both the electroantennogram (EAG) technique and single-cell recordings, whilst a 2-choice pitfall assay and a single-insect arena test were used to investigate behavioural responses.

Food-produced volatiles from carob pods were found to produce large amplitude antennal responses and attraction in behavioural assays. Analysis of such volatiles by coupled gas chromatography-electroantennography (GC-EAG) demonstrated at least 3 active components, of which one was identified as hexanoic acid. Attraction to food odour was found to vary with insect age, and this correlated with similar variation in the EAG response, suggesting a role for peripheral receptor sensitivity in modulating insect behaviour.

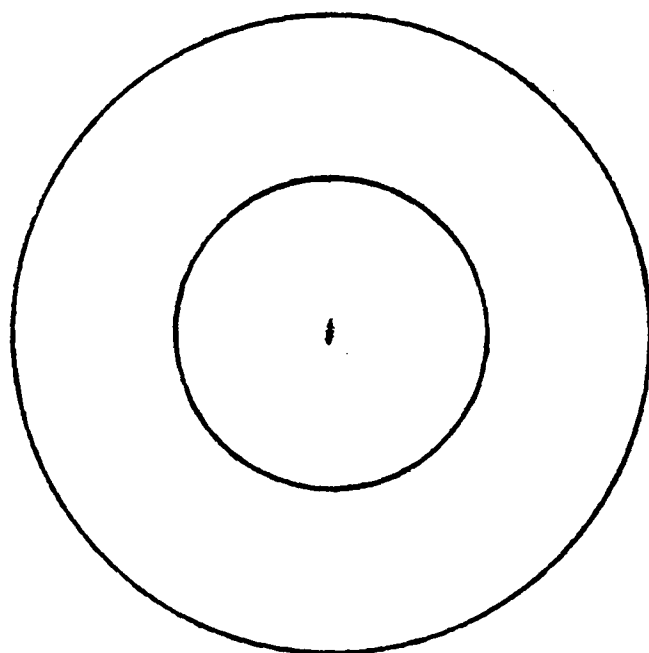
Beetle-produced volatiles also caused antennal responses and attraction of conspecific individuals of both sexes, thus acting as an aggregation pheromone. The active components were identified by GC-EAG as (Z,Z)-3,6-dodecadien-11-olide, (Z,Z)-3,6-dodecadienolide and (Z,Z)-5,8-tetradecadien-13-olide. These act as a multicomponent pheromone, with separate antennal receptors for each component. Although no sexual differences in antennal responses were found, consistently more females than males were attracted to the pheromone. This suggested a partial sex pheromone function, as the pheromone was shown to be male-produced. However, the ratio of females:males attracted was found to vary with the blend ratio tested. Thus if males are able to alter the blend they produce, the pheromone may act at different times as either a sex pheromone or an aggregation pheromone.

Finally, the functional and evolutionary importance of odours to this insect are discussed.

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The Saw-toothed Grain Beetle Oryzaephilus surinamensis.

Life Size.

CHAPTER 1.

INTRODUCTION.

Odours, when sweet violets sicken,
Live within the sense they quicken.

Shelley.

Odours play a critical role in the lives of most insects. They "quicken" the senses of a multitude of species, to an almost equally wide variety of ends. Many such odours are insect-produced, and these may act intraspecifically, i.e. as pheromones, to facilitate the location of mates, co-ordinate courtship, promote aggregations, elicit alarm behaviour or facilitate trail-following, among other things. Numerous examples of such sex pheromones, aggregation pheromones, alarm pheromones and trail pheromones have been documented, and are included in recent reviews (e.g. Tamaki, 1985; Carde and Baker, 1984; Borden, 1985; Birch, 1984; Blum, 1985; Nault and Phelan, 1984; Bradshaw and Howse, 1984).

Odours also figure prominently in interspecific interactions, especially where these involve the location of suitable hosts or food. Many insect predators track down their prey by means of odour cues, and many insect parasitoids and parasites use a similar mechanism to locate hosts (Vinson, 1984). Interactions between insects and plants may also be included in this interspecific category, since a wide variety of phytophagous insects are also known to use specific odour

components to locate their host plants (Visser, 1986). Understandably, this importance of odours to insects has led to considerable interest in the production and perception of olfactory cues.

The olfactory physiologist Jurgen Boeckh recently stated (1986):-

"Insect olfaction represents a problem which can be comprehensively approached only by a combination of aspects and methods in a multidisciplinary effort."

This multidisciplinary effort has involved investigations of the structure and distribution of antennal olfactory sensilla (Zacharuk, 1980; Chapman, 1982), the chemistry of insect and food-produced volatiles (Mori, 1984; Tamaki, 1985), the electrophysiological responses of olfactory receptors (Mustaparta, 1984) and the behavioural responses of the insects themselves (Carde, 1984; Payne et al ., 1986). Each area has been extensively studied, and the reader is directed to the above reviews and the individual chapters of this thesis for further details and examples. Studies of insect olfaction have, however, suffered from two limitations. Firstly, detailed investigations in species other than moths remain relatively few (Schneider, 1986; Tumlinson, 1985). Secondly, as a consequence of the multidisciplinary approach, different aspects of insect olfaction have often been studied in different laboratories and on different species (Birch and Barton-Browne, 1986). As a result, it has not always been possible to determine whether differences observed have reflected species differences, or have been due to the use of different techniques. The specificity of olfactory receptors in bark beetles

provides a good example of this:- In species of the genus Ips , using single cell recordings and low odour concentrations, Mustaparta et al., (1979, 1980) found that each olfactory receptor cell was specific for a single pheromone component. In another bark beetle - Dendroctonus frontalis - however, a different technique ("differential adaptation") led Payne (1975) and Dickens and Payne (1977) to conclude that each receptor cell responded to several pheromone components. This discrepancy could have been due to either species or methodological differences.

Where the same species has been used, each aspect of the olfactory response has often been studied in different laboratories, possibly on separate strains, using animals reared under different conditions. Whilst workers have long realised that factors such as reproductive state, insect age, light intensity and time of day can affect the behavioural response of insects to odours (Adeesan et al., 1969; Shorey and Gaston, 1964, 1965; Shorey et al. , 1968), electrophysiologists have often worked with randomly selected individuals, regardless of such factors (Boeckh, 1986). Recent evidence suggests, however, that the sensitivity of peripheral olfactory receptors may also vary with factors such as age (Dickens, 1986) and state of satiation (Davis, 1984; Warnes and Finlayson, 1986) in a variety of insects. Consequently, meaningful correlations between behavioural and receptor responses, and also with the distribution of olfactory sensilla have not always been possible.

At the most recent international seminar devoted to insect olfaction (Oxford, 1984 - see Payne et al. , 1986), there were several

calls for a multidisciplinary and integrated approach by studying separate aspects and applying different methods to a single insect species (e.g. Birch and Barton-Browne, 1986). The aim of this thesis, therefore, was to attempt this by investigating antennal structure, the chemistry of insect and food-produced volatiles and the receptor and behavioural responses of individuals to such odours in a single species of insect.

The Silvanid beetle Oryzaephilus surinamensis L. (the saw-toothed grain beetle) was used for two reasons. Firstly, several workers have previously shown that this species responds behaviourally to both food and beetle-produced volatiles and, secondly, because of its economic importance. Although originally subcortical in habit and tropical in distribution (Halstead, 1980), O. surinamensis is a world-wide pest of stored grain and processed cereals (Howe, 1956; Loschiavo and Smith, 1970) and the major grain store pest in the U.K. (Freeman, 1976). These beetles not only consume and damage grain, but their presence can lead to rejection of foodstuffs, and thus loss of revenue. Under present U.K. tolerance limits, a consignment of grain can be rejected following the discovery of a single live insect (Pinniger et al. ., 1984), and so there is clearly a need for an effective detection and monitoring system for O. surinamensis and other stored-product insects. Traps containing sources of either food odours (Levinson and Levinson, 1979) or pheromones (Burkholder and Ma, 1985) have been proposed, and thus the aim of previous work (and an additional aim of this study) has been to identify suitable attractant materials.

Several food odours have been shown to cause a behavioural response in O. surinamensis. Extracts from oats (Freedman et al.., 1982; Mikolajczak et al.., 1983, 1984) and rolled oat volatiles (Pierce et al.., 1981) caused aggregation in laboratory assays, and several active components have been identified. Similar responses were found to brewer's yeast volatiles (Pierce et al.., 1981) and also to extracts and volatiles collected from pods of the carob tree Ceratonia siligua (O'Donnell et al.., 1983; Stubbs et al.., 1985).

Individuals have also been shown to respond behaviourally to insect-produced volatiles isolated from both adult beetles and frass (Pierce et al.., 1981), and such responses were further found to vary with the age and density of the beetles tested (Pierce et al.., 1983). More recently, 3 beetle-produced materials have been identified, which act as an aggregation pheromone (Pierce et al.., 1984a, 1985), but the exact function and mode of action of this pheromone remain vague.

However, other aspects of olfaction in this beetle have not previously been addressed. For example, no investigations of the olfactory receptor system have been attempted, with respect to either structure or electrophysiology.

The aims of this work were, therefore, to:

i. Determine the olfactory capability of the antenna of O. surinamensis by investigating the structure, function and distribution of antennal sensilla.

ii. Record the antennal responses to both food and beetle-produced odours, and to determine the factors which affect such responses. This also involved a critical study of the antennal

response.

- iii. Identify the active components in the stimulating odours.
- iv. Investigate the behavioural responses of beetles to odours, and determine the factors that affect such responses.
- v. Investigate the production of volatiles by adult beetles.

As will be discussed in the chapters that follow, this has provided a broad overview of olfactory responses in this one species, and has allowed direct comparisons between observed behaviour, receptor responses and antennal structure. Such studies have also provided a basis for discussion of the evolutionary and functional significance of odours to this beetle.

CHAPTER 2.

FINE STRUCTURE, FUNCTION AND DISTRIBUTION OF ANTENNAL SENSILLA.

INTRODUCTION.

Mechanical, chemical, humidity and thermal receptors are known to exist on the antennae of adult insects, and much previous work has concentrated on elucidating the detailed ultrastructure of such receptors. By studying both ultrastructure and electrophysiology, it has been possible to relate structural features of receptors to function, and extensive reviews now exist of the structures typically found in mechanoreceptors (McIver, 1975), chemoreceptors (Zacharuk, 1980) and thermo / hygrometers (Altner and Loftus, 1985). Antennae from several insect orders have been examined, including Lepidoptera, Hymenoptera, Hemiptera, Diptera, Dictyoptera, Coleoptera and Orthoptera.

Within the Coleoptera, most attention has been directed at the family Scolytidae, and little has been directed at the large number of beetles that inhabit stored-products. Exceptions have included early work (light microscopy) on the antennae of Tribolium castaneum and T. confusum (Roth and Willis, 1951), and more recently, work on receptors in larval T. castaneum (Behan and Ryan, 1978), and on antennae of adult Tenebrio molitor (Harbach and Larsen, 1977). No previous investigations have been made into the antennae of O. surinamensis or of any other members of the family Silvanidae.

The purpose of this work was to describe the structure of the antenna and its sensilla by scanning and transmission electron

microscopy (SEM and TEM), and hence deduce sensillum function by comparison with structures found in other insects. Where possible, sensillum function was confirmed by electrophysiological recordings. To assess the sensory capability of the antenna, and to complement the electrophysiological and behavioural studies reported below, the numbers and distribution of sensilla were mapped by SEM. Complete maps have been obtained for several coleopteran species (Chapman, 1982) and comparisons with this existing data are discussed.

MATERIALS AND METHODS.

Insect material.

Cultures of O. surinamensis were obtained from the Ministry of Agriculture, Fisheries and Food laboratory, Slough Laboratory, and maintained on a 5:5:1 mixture of rolled oats, whole wheat flour and brewer's yeast at 25°C. Beetles used for structural work were 1 - 2 weeks post adult eclosion. Individuals for both SEM and TEM were removed from the diet and kept in clean culture jars for 24 hours before fixation to reduce contamination of the antennae with diet.

SEM procedure.

Individuals were beheaded and the heads either air-dried or critical-point dried. Heads were mounted on stubs using silver paint, sputtercoated with gold, and examined on a Phillips PSEM 500 or Jeol JSM 35 CF microscope.

TEM procedure.

Individual antennae were removed and fixed overnight at 4°C in 2.5% glutaraldehyde in 0.05M cacodylate buffer with 5% sucrose, buffered at pH 7.4.

Specimens were washed in buffer with several changes, before immersing in 1% osmium tetroxide for one hour. After dehydration through ethanols and embedding in Araldite, thin sections were cut using a diamond knife on a Reichert OM U4 ultramicrotome and supported on Formvar coated grids. Contrast was enhanced using uranyl acetate and lead citrate, and the sections examined on a Phillips 400T electron microscope.

Measurements.

Distributions of sensilla were obtained by photographing dorsal, ventral and terminal views of antennae. Sensilla in pits on segments 9 and 10 were counted by removing one or two segments and photographing the exposed tip. A minimum of six individuals was used for each view, for each sex.

Sensilla lengths were measured from a minimum of 20 sensilla, taken from at least 6 individuals. Sensilla diameters were measured from both SEM and TEM pictures.

Classification of sensilla.

Several different classifications of insect sensilla have been used in the past. Throughout this thesis the scheme suggested by Altner (1977) and later amended by Zacharuk (1980) is used, which relies on details of the internal structure of the sensillum, and the presence or absence of pores.

Recording techniques.

Recordings were made from the UP sensilla, using the tip recording technique. Adult beetles were mounted ventral surface uppermost on a cork stage, using double sided tape. The antennae were restrained with a thin band of tape half-way along their length. Under 320x binocular magnification the indifferent electrode was inserted into the mouth. The recording electrode containing the test solution was brought up to contact the tip of the UP sensillum for 5s. and the responses recorded. Recordings were made using a Grass P-16 pre-amplifier in single ended mode, displayed on a Tectronix 3223 CRO,

and recorded on a Racal Store 4 tape recorder. Permanent traces were made using a Medelec UV recorder, from which measurements and counts were made.

Individual UP sensilla were identified and the response of each sensillum to a range of sodium chloride concentrations tested. Solutions were presented in ascending order of concentration, allowing a minimum of 5 minutes between tests for the receptors to recover. A total of 12 sensilla from 3 different individuals were tested.

Mechanoreceptor responses were recorded by placing a recording electrode containing 50mM NaCl over the tip of the UP sensillum, allowing the phasic response to subside, and then stimulating the sensillum by gentle movement through c. 10 degrees in various directions.

All recordings were made between 1400 and 1800 hours.

On the basis of spike amplitude it was difficult to distinguish the activity of individual cells. The results are therefore presented as total spike counts in the initial second of stimulation.

Preliminary single cell recordings (using etched tungsten microelectrodes) were made from sensilla in the lateral pits of segment 10 in response to olfactory stimuli. This work was conducted at the Rothampsted Experimental Station using the experimental set-up of Dr. L.J. Wadhams. The reference electrode was placed in the mouth and the recording electrode was manipulated to penetrate the cuticle around the bases of sensilla in the lateral pits until spikes from single units were detected. Responses were amplified and displayed on a CRO and recorded using an ink jet recorder. On the basis of spike amplitude, it was possible to distinguish the activity of individual

cells. The olfactory stimuli tested included three synthetic pheromone components (II,III and IV) and a food odour (carob distillate) - for further details see Chapters 4 and 5. Odour presentation was essentially similar to that described for EAG studies in chapter 3 (Fig. 12).

RESULTS.

The antenna of Oryzaephilus surinamensis consists of 11 segments, of which the distal 3 segments are enlarged to form a club (Figs. 1, 2a). SEM studies showed the presence of sensilla on all 11 segments, with an increase in density and diversity on the three club segments. Detailed SEM and TEM examination distinguished seven morphologically different sensilla types (Figs. 2,3):

1. non-porous hairs (NPH)
2. non-porous pegs with inflexible sockets (NPP-is)
3. non-porous pegs with flexible sockets (NPP-fs)
4. uniporous sensilla (UP)
5. multi-porous pegs (MPP)
6. multi-porous grooved pegs with single walls (MPG-sw)
7. multi-porous grooved pegs with double walls (MPG-dw)

The distribution of each type is shown in Table 1. There were no significant differences between left and right antennae, nor between males and females.

1. Non-porous hairs (NPH) (Fig.4).

NPH sensilla are the most numerous type on all antennal segments. The cuticular shaft is 16 - 40 μm long (mean=28 μm), tapering from a basal diameter of 1.4 - 3.2 μm to a fine point. Both length and thickness of sensilla decrease on more distal antennal segments. The shaft is ribbed longitudinally, with 6-7 ridges shown in cross-sections (Fig.4b). At its base the shaft becomes circular in cross section, and enters a flexible socket in the cuticle (Fig.4c).

The lumen of the shaft is narrow and non-innervated (Fig.4b).

TABLE 1. Distribution of sensilla types.

Type	Segment										
	1	2	3	4	5	6	7	8	9	10	11
NPH	31 (30-32)	36 (28-45)	26 (22-32)	21 (13-26)	20 (16-26)	24 (19-29)	25 (18-32)	31 (27-36)	71 (61-82)	94 (73-113)	144 (90-199)
NPP-is											3 (1-6)
NPP-fs									1 (0-2)	2 (1-2)	4 (3-7)
UP									8	8	13
MPP									25 (22-29)	63 (56-75)	87 (62-122)
MPG-sw									?	10 (7-12)	?
MPG-dw									0 (0-2)	2 (0-3)	4 (2-8)

Results are means (with range) of six male and six female antennae.

Innervation of the NPH sensillum consists of a single neurone, whose dendrite ends in a well developed tubular body. This is ensheathed in a scolopale, which, in several sections, can be seen to insert excentrically into the base of the shaft (Figs.4d,e)

2. Non-porous pegs with inflexible sockets (NPP-is) (Fig.5).

This is the least numerous type, found sparsely distributed on the tip and outer dorso-lateral surface of the terminal (11th) antennal segment. The sensillum is conical, 5-7um long, with a basal diameter of 1.8 - 2.5 um (Fig.5a). The base is slightly swollen and similar to the bases of the MPP and MPG sensilla.

In transverse sections, the NPP-is is circular with a smooth surface (Fig.5b). For three quarters of its length the shaft is not innervated, and shows no evidence of pores. In the basal quarter, however, the shaft lumen is filled with a single dendrite, containing distinct and regularly arranged microtubules (Fig.5c). In some sections the tubules appeared to be joined by arms of electron-dense material. The dendrite (dia. c. 250nm) penetrates the antennal cuticle below the sensillum (Fig.5d), and then increases in diameter (c. 500 nm) and becomes surrounded by a thick sheath (Fig.5e).

3. Non-porous pegs with flexible sockets (NPP-fs) (Fig.6).

NPP-fs sensilla occur sparsely on the 11th segment, and also singly in the lateral pits on the 9th and 10th segments (Fig.6a). They arise from sunken sockets in the cuticle which appear to allow slight flexibility. They differ from NPH sensilla by their shorter length (5-10 um) and by the absence of longitudinal ridges. The shaft tapers gently from a basal diameter of 1.0 - 1.4um to a blunt tip; in some

instances the tip is slightly swollen. The shaft is circular in cross-section and shows no sign of pores. In some tip sections thin longitudinal grooves are visible, but they do not connect with any internal structures (Fig.6b).

Within the shaft lumen, but ending below the tip, two dendrites are visible (dia. c. 500nm), each containing a dense array of microtubules (Fig.6c). The dendrites completely fill the lumen, and are surrounded by a thick electron-dense sheath. Below the level of the antennal cuticle a third dendrite is visible (Fig.6d). Deep below the cuticle all three dendrites are surrounded by a common dendritic sheath (Fig.6e), but closer to the surface this third dendrite becomes convoluted, and is ensheathed separately.

4. Uniporous sensilla (UP) (Fig.7).

UP sensilla are long sensilla chaetica, found in four distinct rings on segments 9,10 and 11. (Two rings on seg.11, one each on segs. 9 and 10). They are 28 - 48 μm long (mean = 38.5 μm) (the shortest being those on the distal tip of segment 11) and arise almost perpendicularly from the cuticle surface, so standing clear of all other sensilla. The shaft tapers slightly from its base (dia. 1.8 - 2.5 μm) to a blunt tip, and is more strongly ribbed than in NPH sensilla (Fig.7b), with 6-8 rounded ridges seen in section. As in NPH sensilla, the shaft loses these ridges near the base, and enters a large flexible socket in the cuticle (Fig.7c).

UP sensilla are innervated by 6 neurones. The dendrite from one terminates in a tubular body at the base of the shaft. The other five produce unbranching dendrites which enter the lumen and run the whole

length of the shaft (Fig.7b,c). Also present in the lumen of the sensillum is an electron-dense body, which is possibly an extension of one of the sheath cells. The cuticle walls of the shaft are thick, (c. 600 nm at mid-length) and no evidence of wall-pores has been found. SEM studies of the tip, however, have frequently shown a drop of viscous material, which may represent an exudate from a terminal pore. The existence of such a pore was confirmed by electrophysiological recordings (Figs. 11a,b).

5. Multi-porous pegs (MPP) (Fig.8).

MPP sensilla are basiconic pegs found on the segments of the club. On segments 9 and 10 they occur in two lateral pits on the distal surface of each segment. On segment 11 they occur on the distal half, being more numerous on the outer dorsolateral surface.

They are 8 - 15 μm long (mean 12 μm) , arising from a raised, dome-shaped base 1.8 - 2.8 μm in diameter. The shaft tapers very slightly to a blunt tip (Fig.8a).

Each sensillum contains one or, less commonly, two neurones (Fig.8f), whose dendrites branch within the lumen of the shaft. Transverse sections show a range of up to 27 dendritic branches (Figs.8d,e), with microtubules in each dendrite. The cuticular walls of the shaft are thin (100 - 140 nm), and transverse and longitudinal sections show the occurrence of numerous pores (Figs.8b,c,d,e), which connect with the dendrites, via pore kettles and pore tubules. The pores are distributed evenly over the sensillum, except near the base, where no pores are present. Pore density is c. 20 pores per square μm .

6. Multi-porous grooved pegs with single walls (MPG-sw) (Fig.10).

This type was only positively identified in small numbers in the pits on segment 10 (Fig.3b). They may occur on segments 9 and 11 but in the SEM they are similar to MPP sensilla, with a raised base, but with a generally shorter shaft and a swollen, rounded tip. MPG-sw are easily distinguished in transverse sections, as longitudinal grooves (13-17) are visible in the swollen region (Fig.10), though not nearer the base. There was evidence of pores leading from the grooves into the lumen of the shaft, which contains the dendrites. The material did not allow for a detailed analysis of the innervation of this type.

7. Multi-porous grooved pegs with double walls (MPG-dw) (Fig.9).

MPG-dw sensilla are found sparsely distributed on the tip of segment 11, mainly on the dorsal surface, and in the pits on segments 9 and 10. They are short basiconic sensilla, 6-8 μm in length, arising from raised, dome-shaped bases 2.3-2.7 μm in diameter. In the SEM they can be distinguished from other sensilla by their swollen and pointed tips (Fig.9a). The shaft has a diameter of 0.4-0.65 μm , which increases near the tip to 0.7-0.95 μm . In this swollen region, clear longitudinal grooves are visible in the SEM. Transverse sections of the shaft show it is circular in section for most of its length, becoming polygonal (heptagonal or octagonal) and then stellate in the grooved region (Figs.9b,c,d). Seven or, more commonly, eight grooves are present.

MPG-dw sensilla are innervated by two to five neurones (Fig.9e). The dendrites from these cells are unbranched and run the length of

the shaft to the grooved region within a cuticular sheath, and are thus separated from the main lumen of the sensillum. The dendrites are connected to the exterior in the swollen region by radial spoke canals (length c. 120nm, dia. c. 40nm.) opening from the grooves. These canals appear to connect directly with the surface of the dendrites; there is no evidence of pore kettles or tubules. In several SEM specimens drops of an exudate appeared to have emanated from pore openings in the grooves.

Electrophysiological recordings.

Tip recordings demonstrated the activity of the neurones within the UP sensilla, thus confirming the existence of a terminal pore. Results showed that the total impulse frequency in the first second of stimulation increased with increasing concentration of NaCl from 0.01M to 1.0M (fig. 11a). The dose-response curve suggests that the maximum response had not been reached, even at 1.0M NaCl. The relatively high response, even at the lowest concentrations may partly reflect the initial phasic response to mechanical stimulation by the recording electrode. Deliberate mechanical stimulation of the UP sensillum resulted in rapid firing of a neurone giving large amplitude spikes (fig. 11b), demonstrating a mechanosensory function.

Responses of 9 cells from the lateral pit of segment 10 were recorded. Of these, 7 responded to olfactory stimuli (3 to synthetic pheromone components and 4 to carob distillate). One cell responded phasically when the fibre optic lamp trained on the preparation was switched on, presumably in response to the light or heat produced. The other cell did not respond to any of the applied stimuli.

These preliminary results thus demonstrate the preponderance of olfactory receptors in the pit area of segment 10, although it was not possible to associate particular responses with morphologically identifiable sensilla.

TABLE 2. Possible sensilla functions.

Type	Total	Neurones/ Sensillum	Pores	Possible functions
NPH	523	1	-	Mechanoreceptor
NPP-is	3	1	-	Hygroreceptor? Mechanoreceptor?
NPP-fs	7	3	-	Hygroreceptor Thermoreceptor
UP	29	6	1 (terminal)	Contact chemoreceptor
MPP	175	1-2	Many (wall-pores)	Olfactory chemoreceptor
MPG-sw	10	?	Many (wall-pores)	Olfactory chemoreceptor
MPG-dw	6	2-5	Many (wall-pores)	Olfactory chemoreceptor

EXPLANATION OF FIGURES 1 - 10.

ABBREVIATIONS USED:-

b Electron-dense body	pc Pore canal
c Cuticle	pk Pore kettle
cs Cuticular sheath	s Shaft
d Dendrite	sc Scolopale
db Dendritic branch	sh Sheath cell
ds Dendritic sheath	sn Sensillar channel
g Groove	sp Spoke canal
m microtubule	ss Sensillar sinus
p Pore	tb Tubular body

SENSILLA TYPES.

- 1 Non-porous hairs (NPH).
- 2 Non-porous pegs with inflexible sockets (NPP-is).
- 3 Non-porous pegs with flexible sockets (NPP-fs).
- 4 Uniporous sensilla (UP).
- 5 Multiporous pegs (MPP).
- 6 Multiporous grooved pegs with single walls (MPG-sw).
- 7 Multiporous grooved pegs with double walls (MPG-dw).

- FIG.1. O. surinamensis antenna, ventral view. Scale = 100um.
- FIG.2 a. Terminal 3 club segments of antenna, dorsal view, showing position of lateral pits on segments 9 and 10 (arrows). Scale = 100um.
b. Dorsal view of antennal tip. Scale = 10um.
- FIG.3 a. Dorsal surface of segment 11 showing different sensilla types. Scale = 10um.
b. Lateral pit of segment 10. Scale = 10um.
- FIG.4. Non-porous hairs (NPH).
a. NPH hairs on distal end of segment 10. Scale = 10um.
b. T.S. of NPH sensillum, showing lack of innervation in shaft. Scale = 0.5um.
c. T.S. of NPH through socket region. Scale = 0.5um
d. T.S. of NPH below level of cuticle, showing tubular body. Scale = 0.5um.
e. Oblique L.S. through base of NPH sensillum. Scale = 0.5um.
- FIG.5. Non-porous pegs with inflexible sockets (NPP-is).
a. NPP-is sensillum (2) on segment 11. Scale = 5um.
b. T.S. of NPP-is sensillum near tip. Scale = 0.5um.
c. T.S. of NPP-is sensillum near base, with dendrite in shaft lumen. Scale = 0.5um.
d. T.S. of NPP-is base showing dendrite penetrating cuticle. Scale = 0.5um.
e. T.S. of NPP-is base below level of cuticle, showing single dendrite within sheath. Scale = 0.5um.
- FIG.6. Non-porous pegs with flexible sockets (NPP-fs).
a. NPP-fs sensillum in pit on segment 10. Scale = 10um.
b. T.S. of NPP-fs sensillum near tip showing thin grooves, but lack of dendrites. Scale = 0.5um.
c. T.S. of NPP-fs sensillum as it emerges from the cuticle. Scale = 0.5um.
d. T.S. of NPP-fs base showing 3 dendrites, one ensheathed separately. Scale = 0.5um.
e. T.S. of NPP-fs base below level of (d), showing all 3 dendrites in a common sheath. Scale = 0.5um.
- FIG.7. Uniporous sensilla (UP).
a. UP sensillum on segment 10. Scale = 10um.
b. T.S. of UP sensillum showing dendrites and electron-dense body in shaft lumen. Scale = 0.5um.
c. T.S. of UP sensillum in socket region. Scale = 0.5um.
d. T.S. of UP base showing tubular body and dendrites in sheath. Scale = 0.5um.
e. T.S. of UP base below level of (d) showing ciliary regions of the 6 dendrites. Scale = 0.5um.
f. Oblique L.S. through UP base. Scale = 0.5um.

FIG.8. Multiporous pegs (MPP).

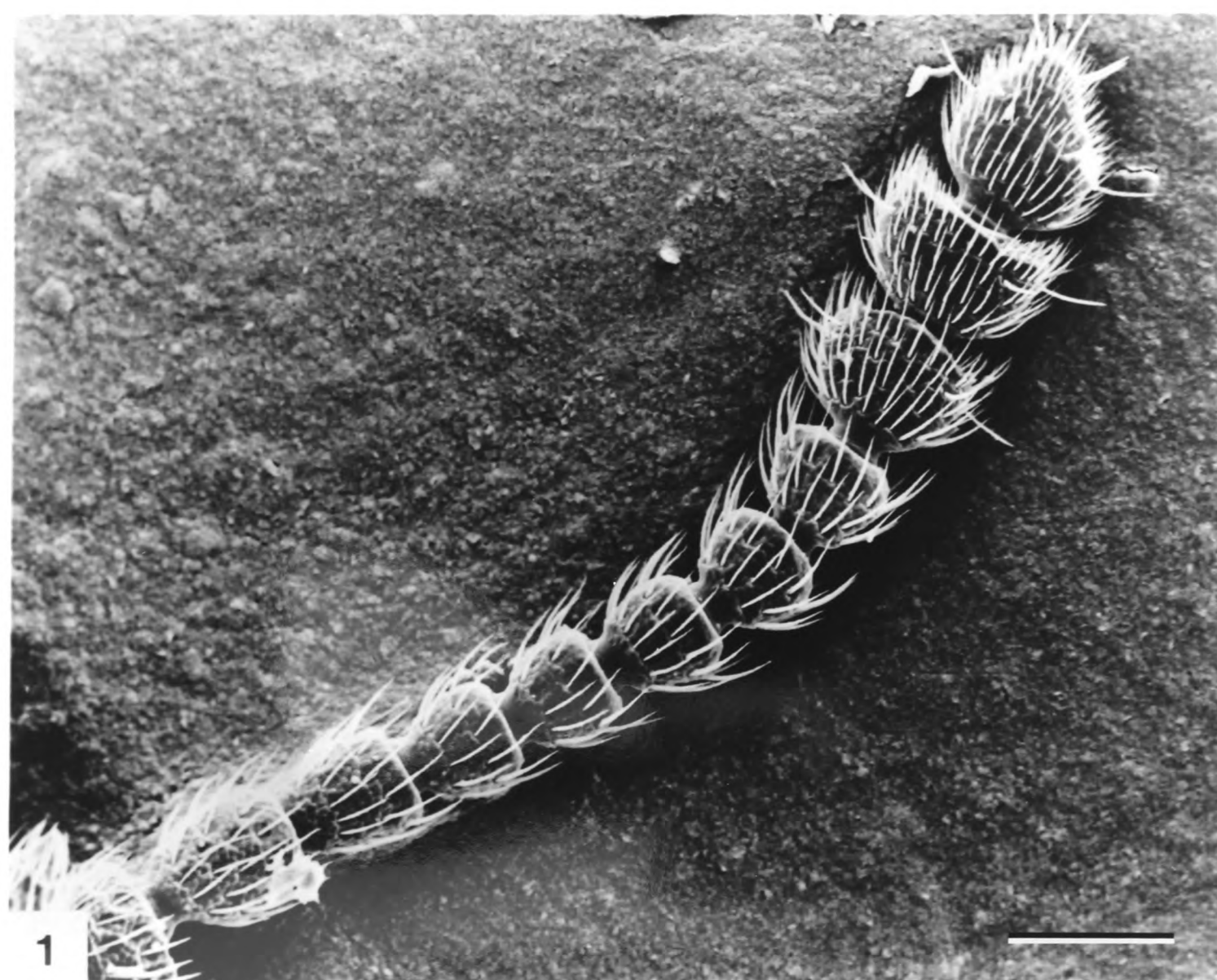
- a. MPP sensillum in pit on segment 10. Scale = 0.5um.
- b. Surface L.S. of MPP sensillum showing pores in plan view. Scale = 0.5um.
- c. L.S. of MPP sensillum showing pores and dendritic branches. Scale = 0.5um.
- d. T.S. of MPP sensillum showing dendritic branches, each containing one microtubule. Scale = 0.5um.
- e. T.S. of MPP sensillum showing numerous pores and dendritic branches. Scale = 0.5um.
- f. T.S. through base of MPP sensillum showing a dendrite surrounded by a sheath cell. Scale = 0.5um.

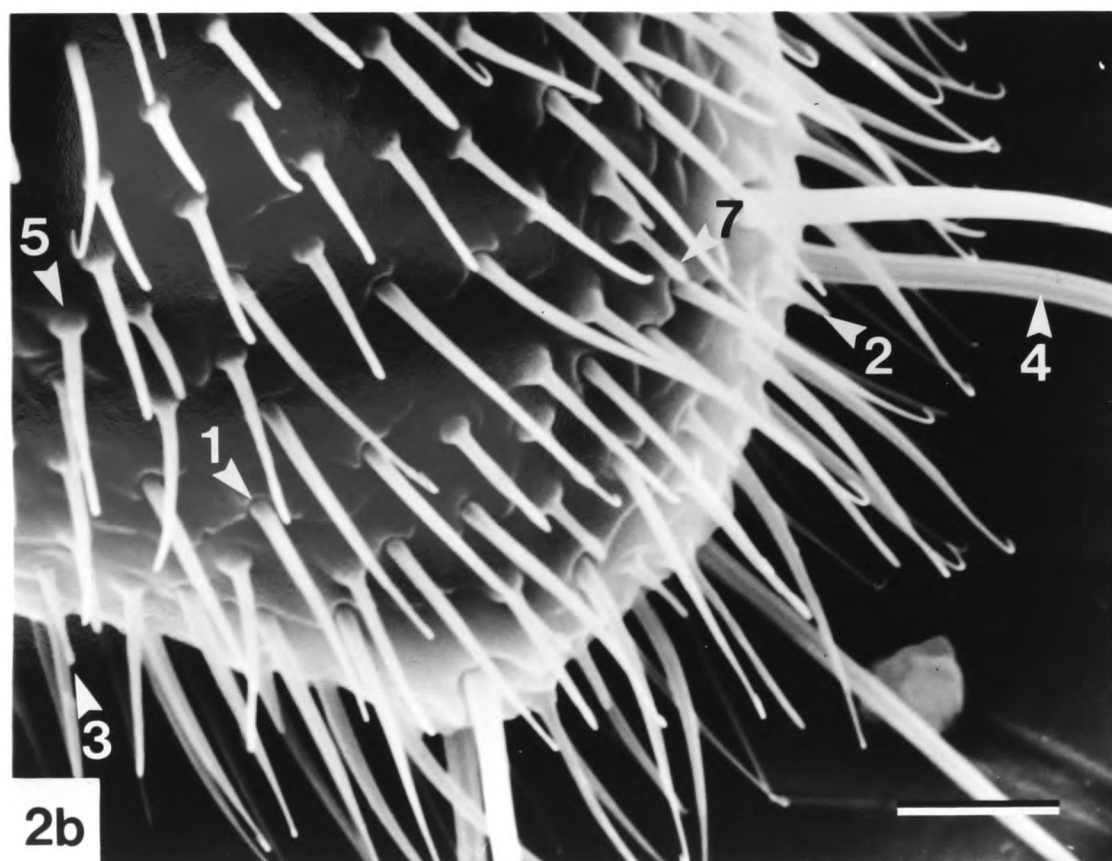
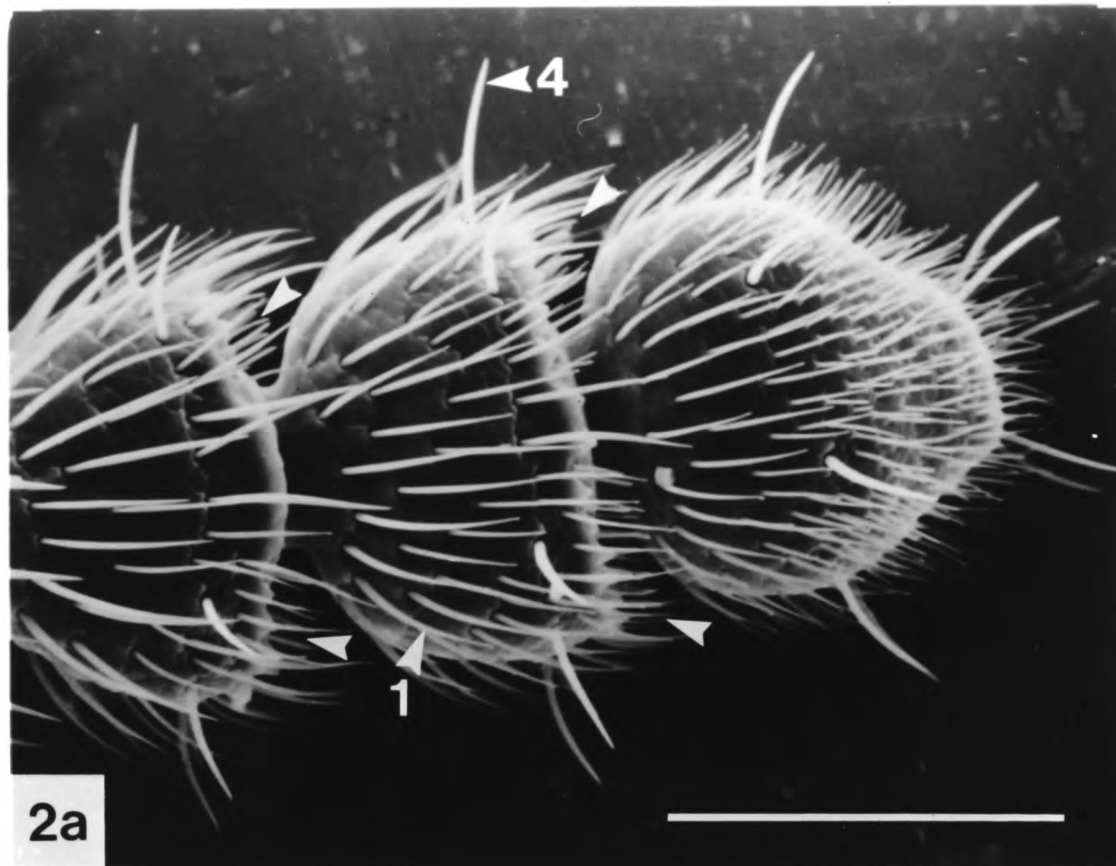
FIG.9. Multiporous grooved pegs with double walls (MPG-dw).

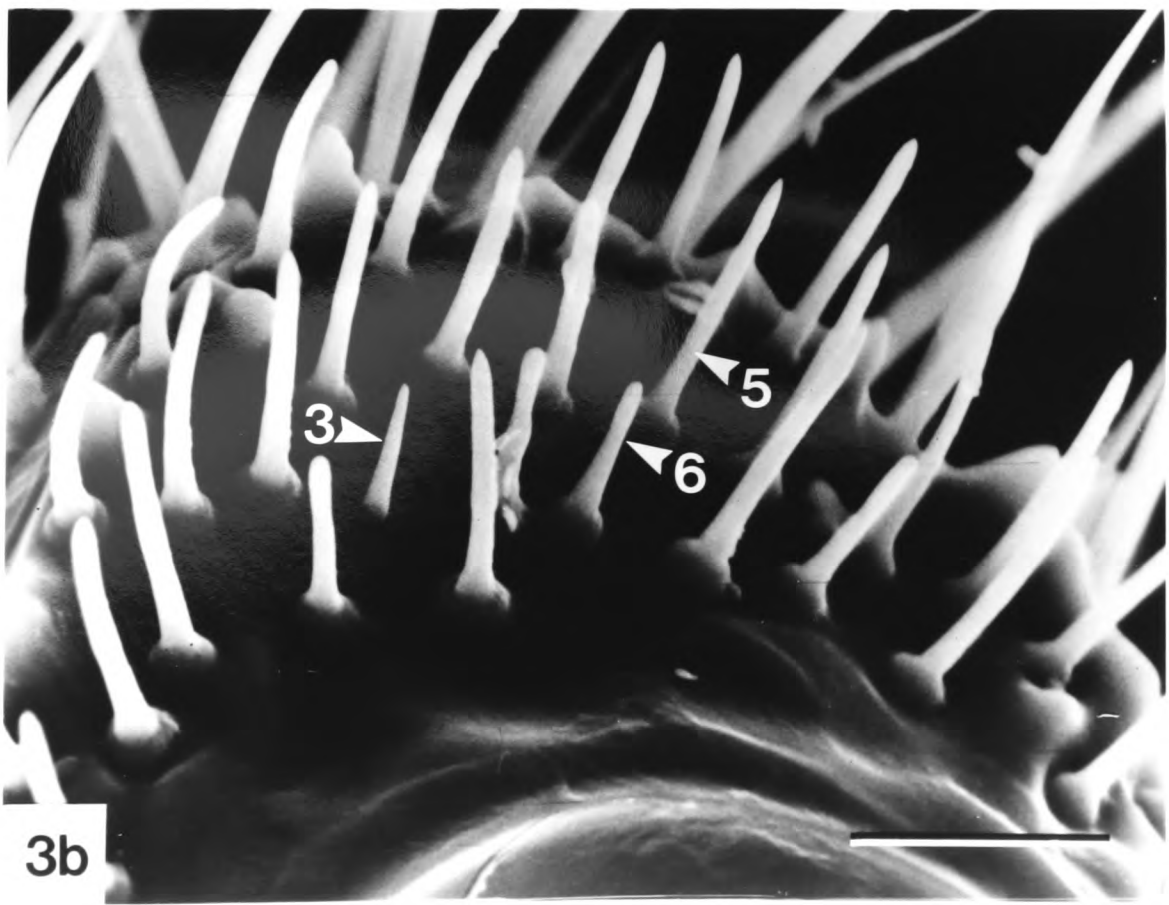
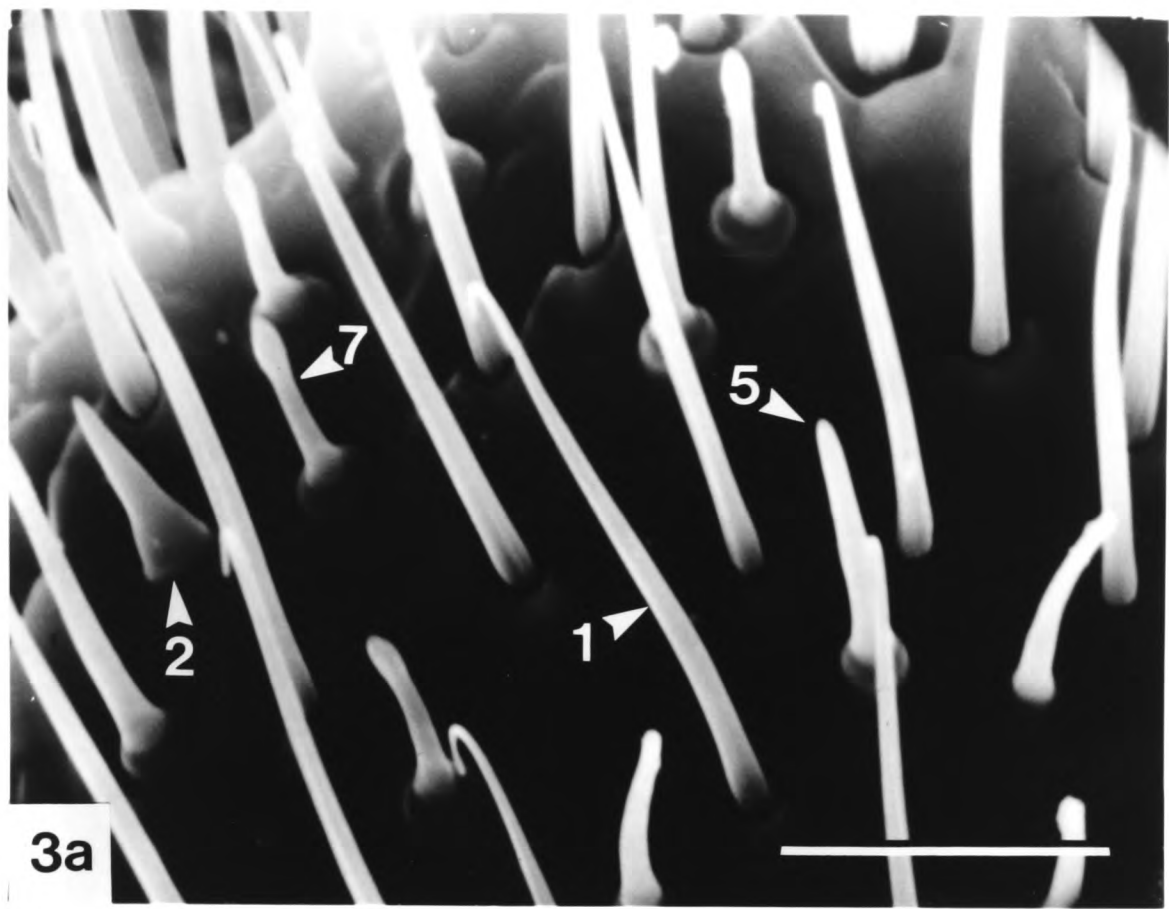
- a. MPG-dw sensillum on segment 11, showing swollen and grooved tip. Scale = 0.5um.
- b. T.S. through grooved tip of MPG-dw sensillum, showing spoke canals leading into the dendrites. Scale = 0.5um.
- c. T.S. through MPG-dw sensillum below grooved region showing polygonal outline. Scale = 0.5um.
- d. T.S. through MPG-dw sensillum below (c) showing double lumen with a sensillar channel. Scale = 0.5um.
- e. T.S. through MPG-dw base below cuticle level, with 5 dendrites surrounded by a cuticular sheath within the sensillar sinus. Scale = 0.5um.

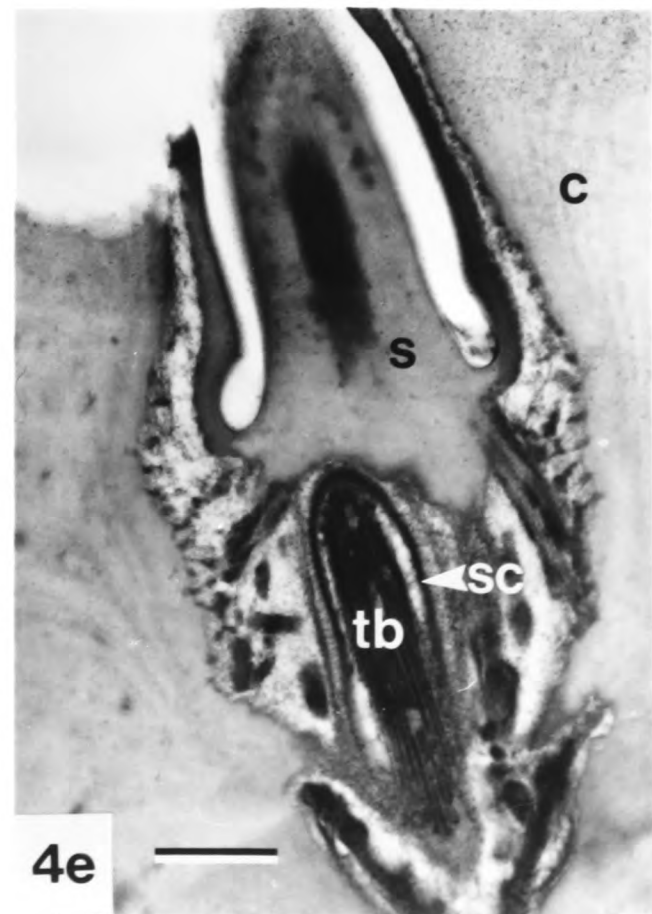
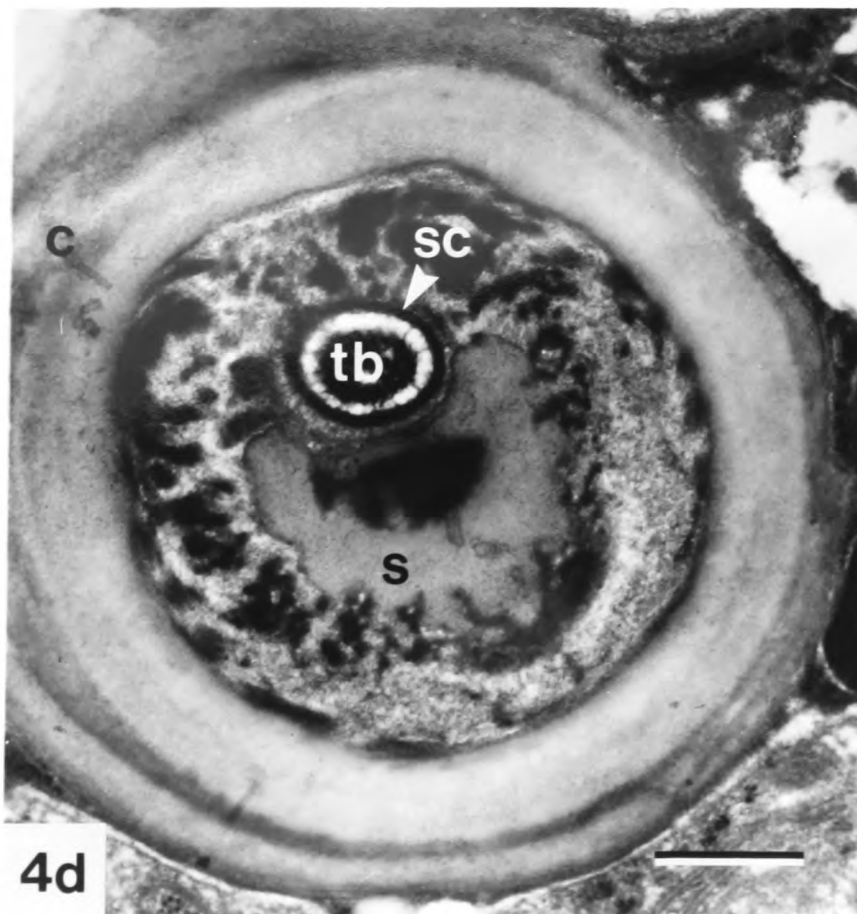
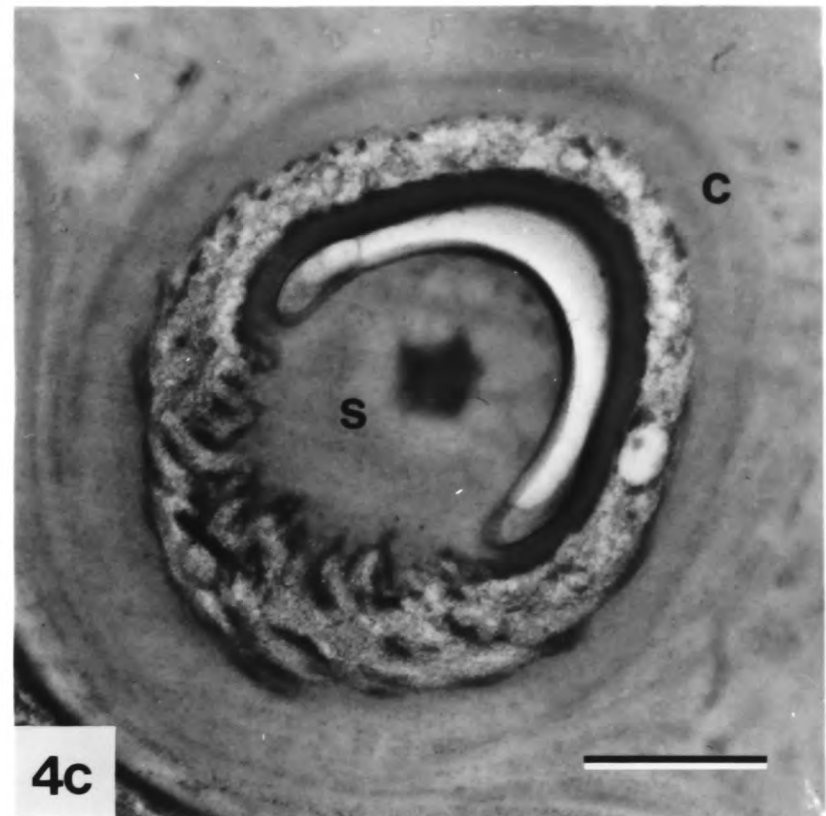
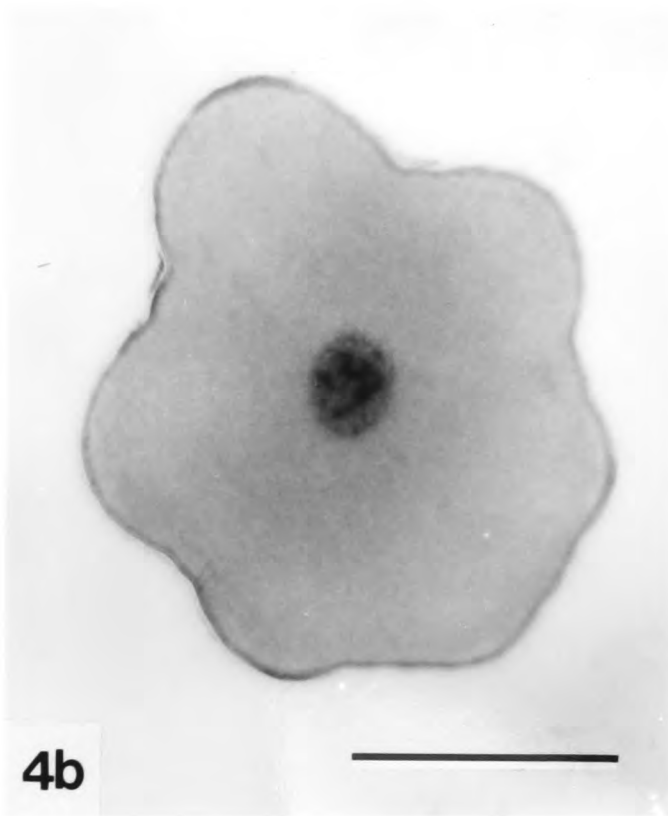
FIG.10. Multiporous grooved peg with a single wall (MPG-sw).

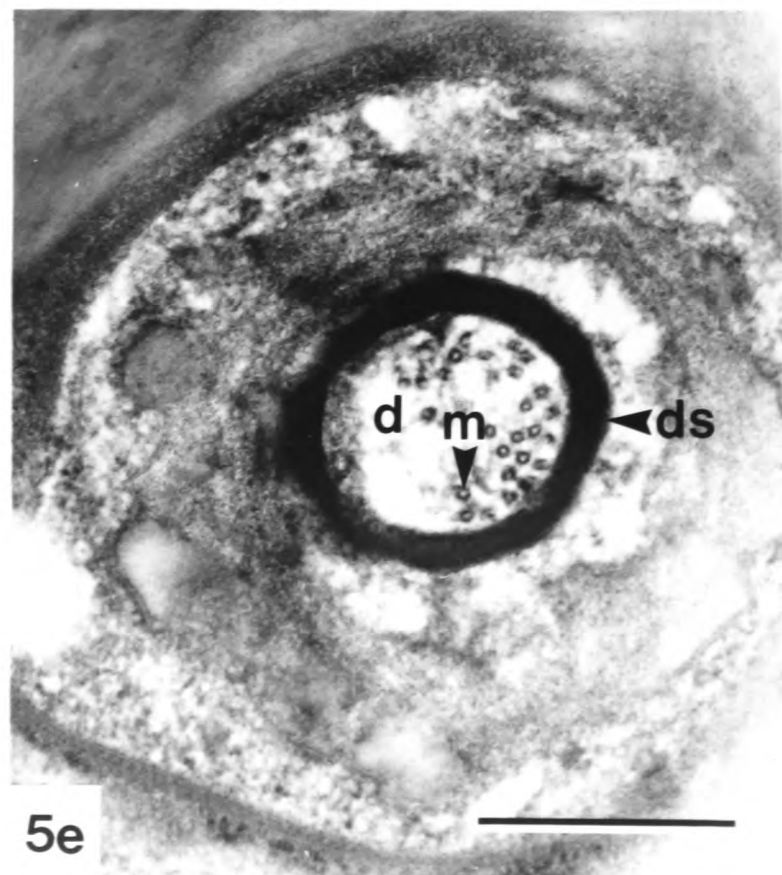
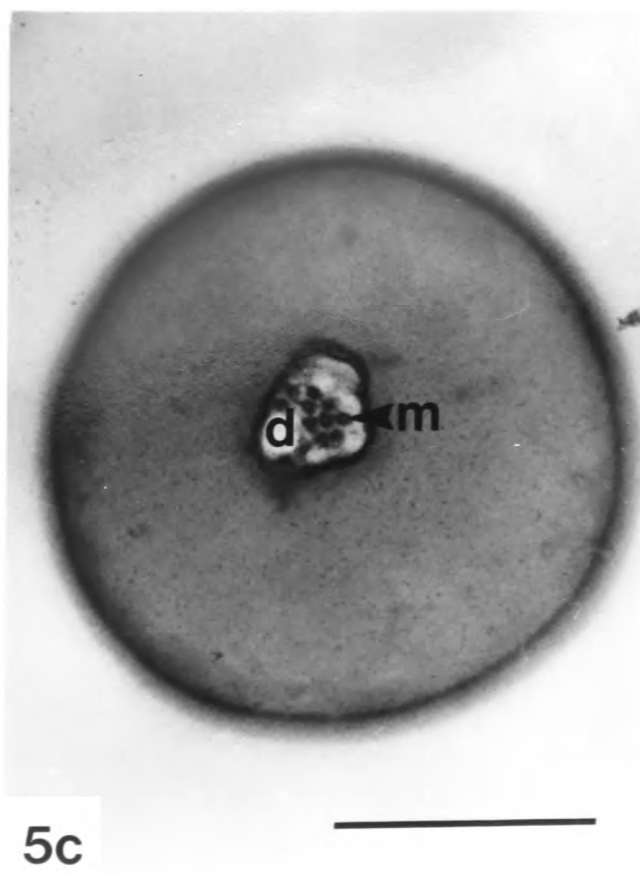
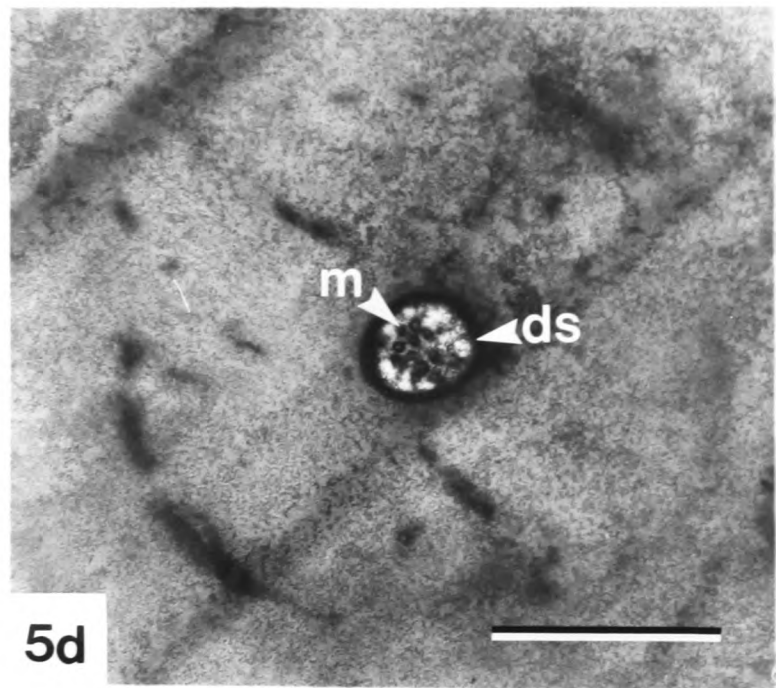
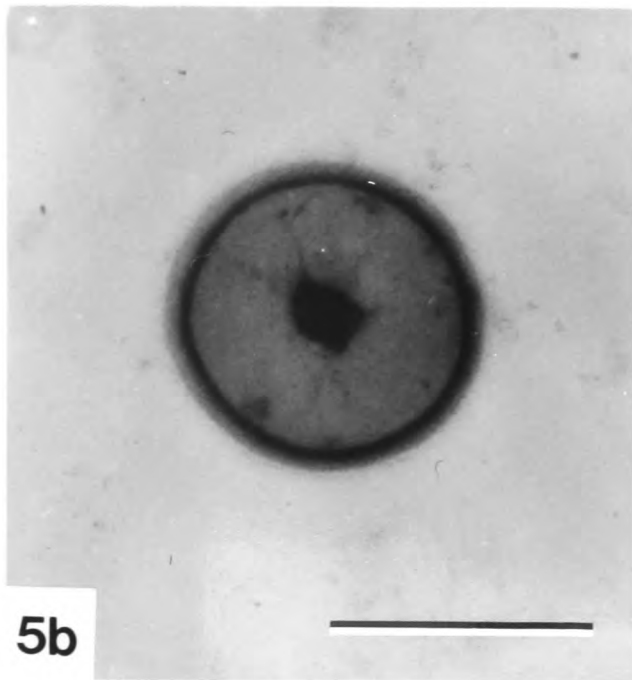
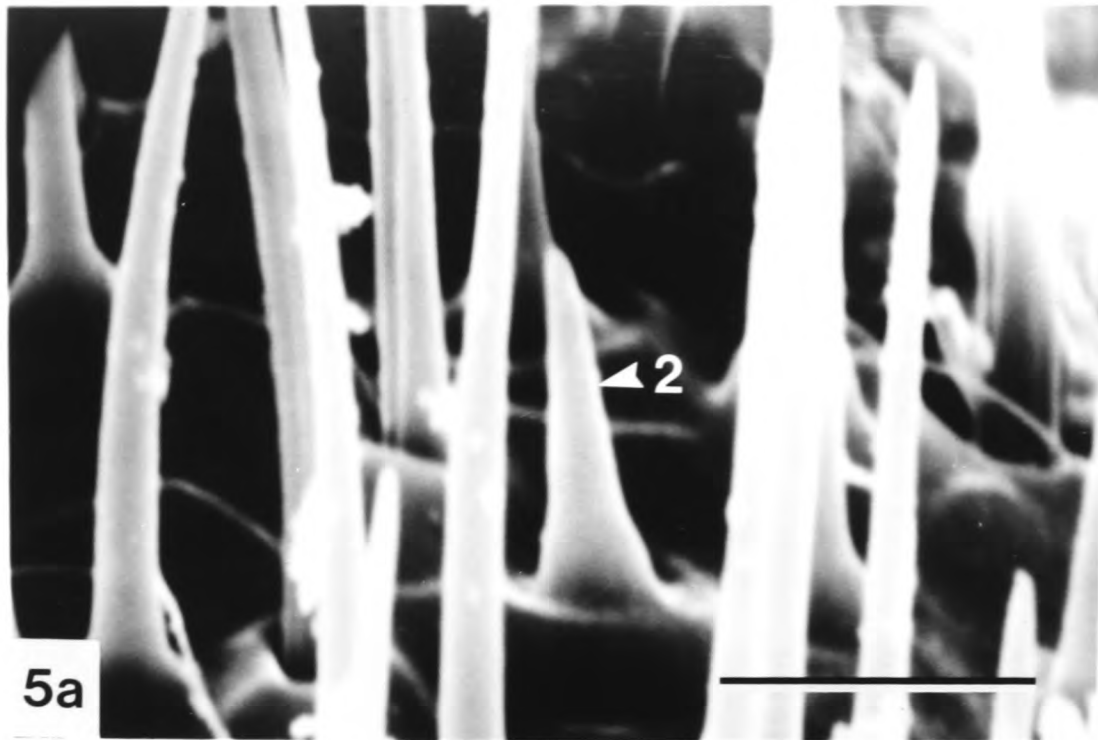
- T.S. through grooved tip region. Scale = 0.5um.

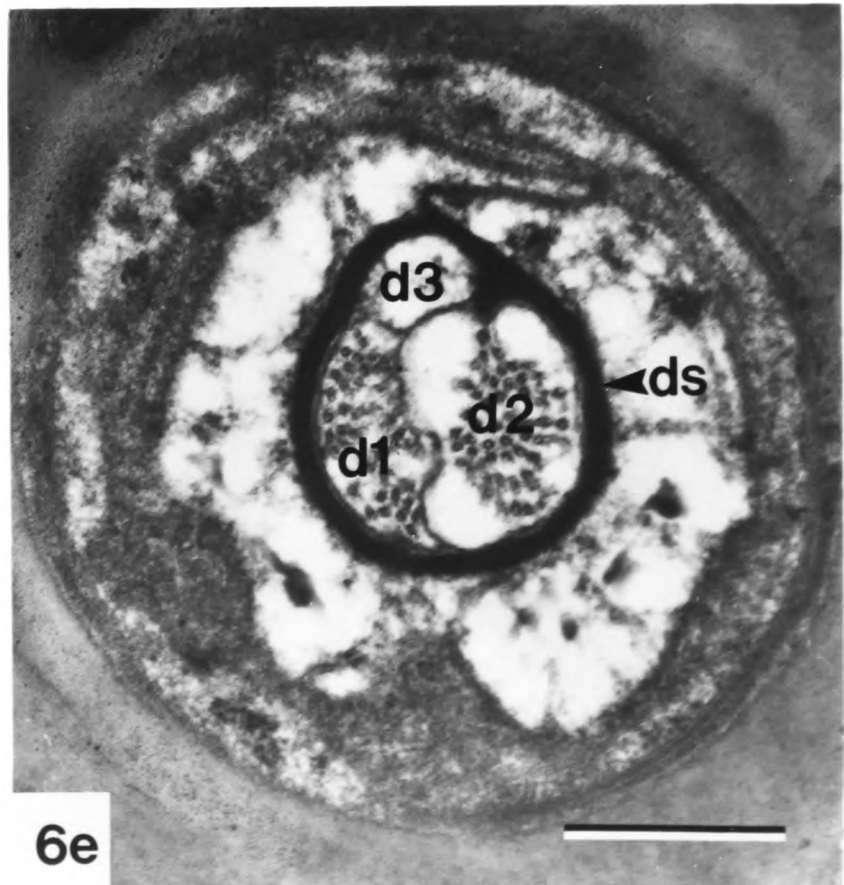
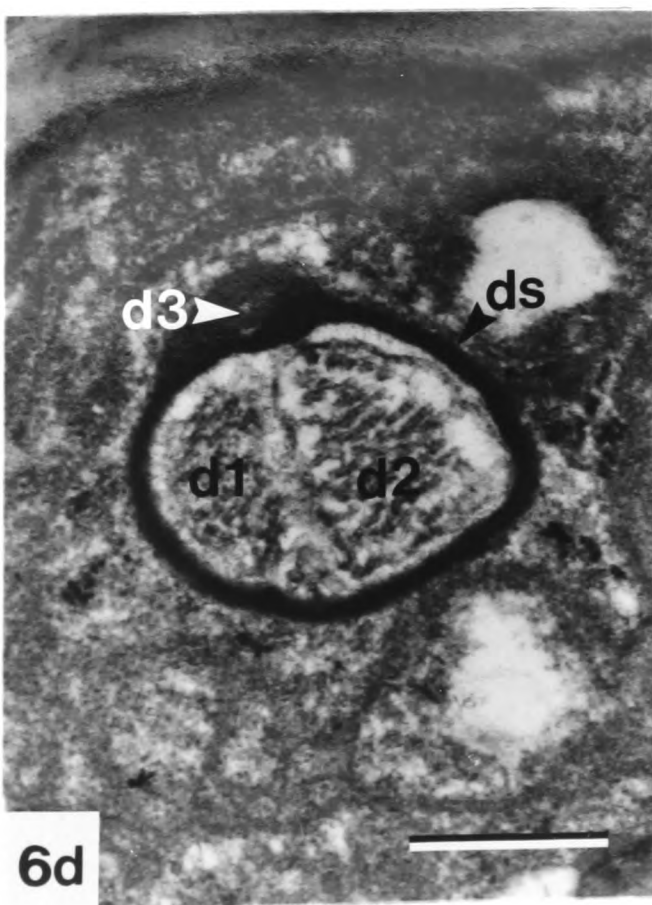
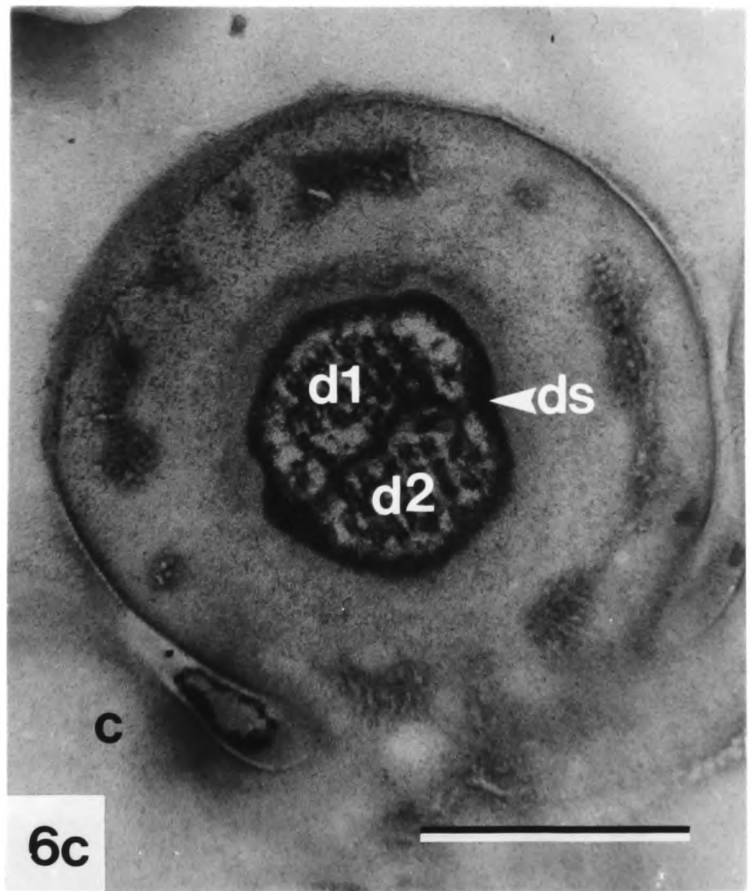
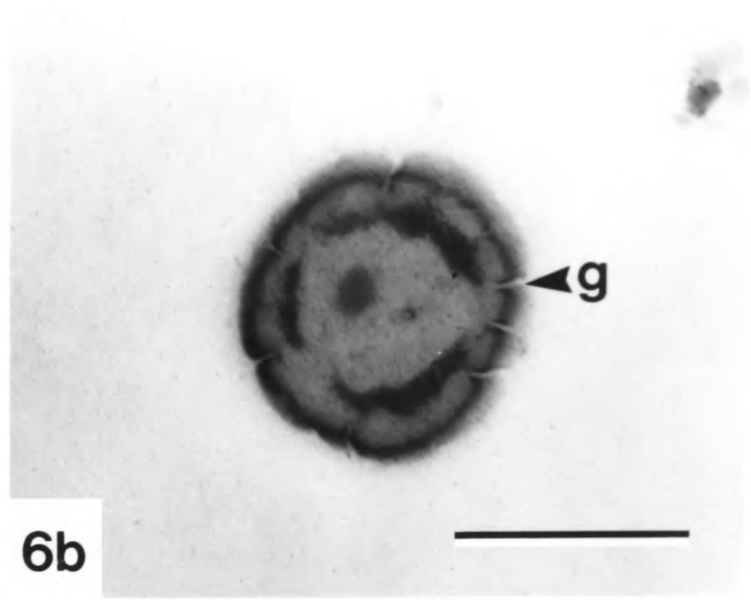
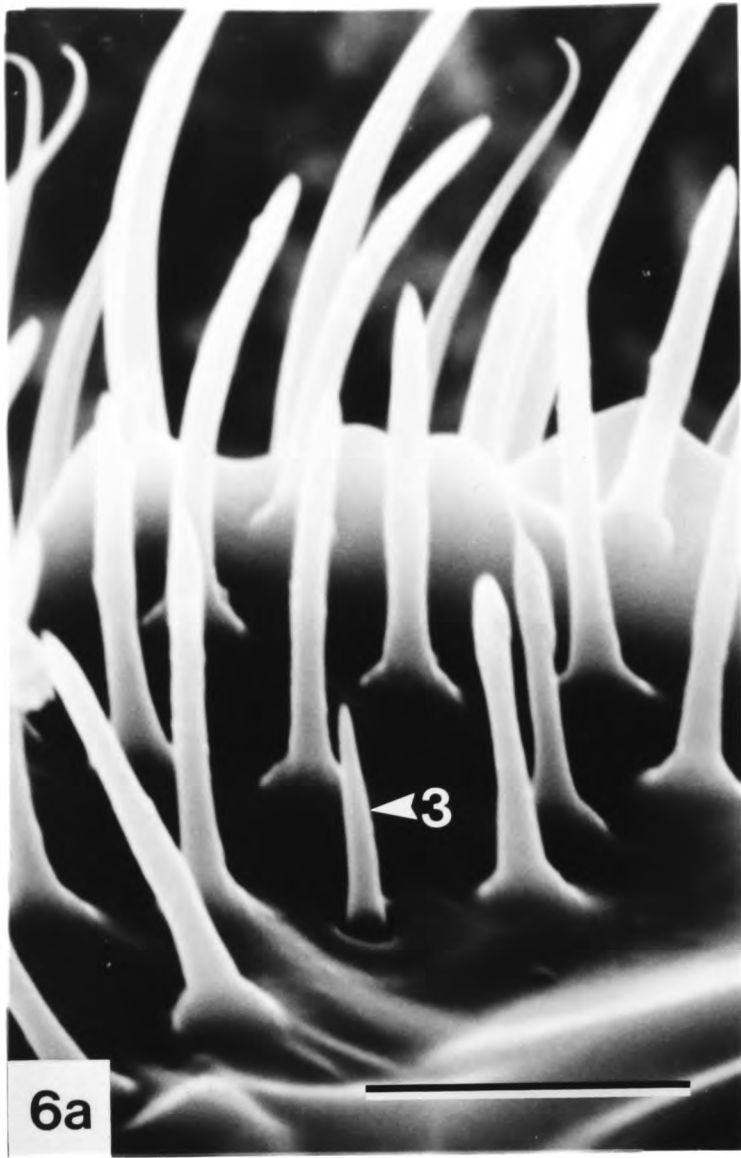


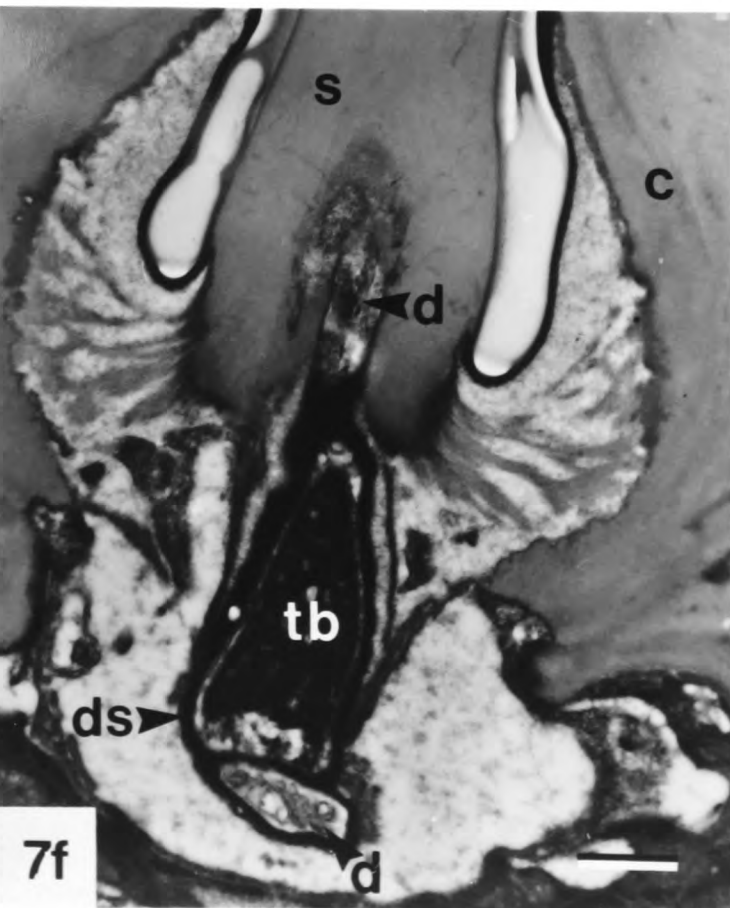
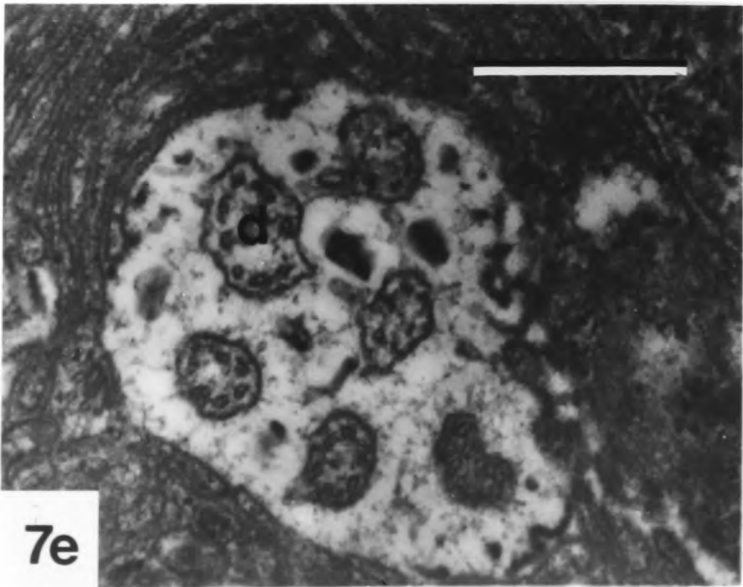
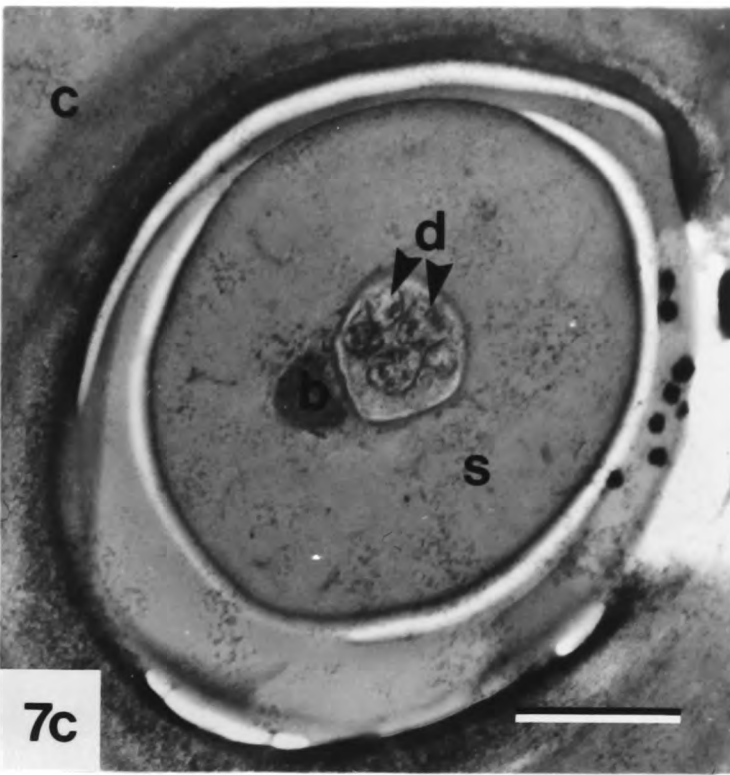
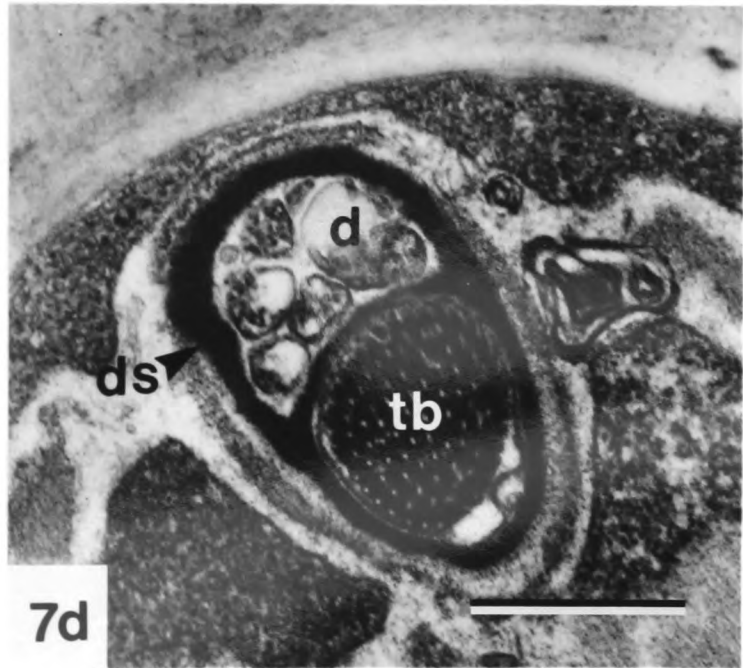
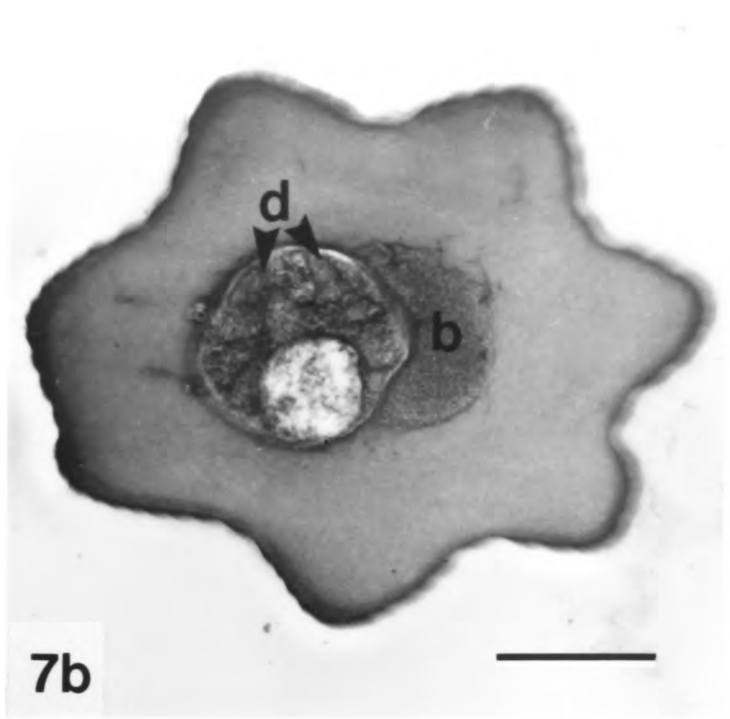
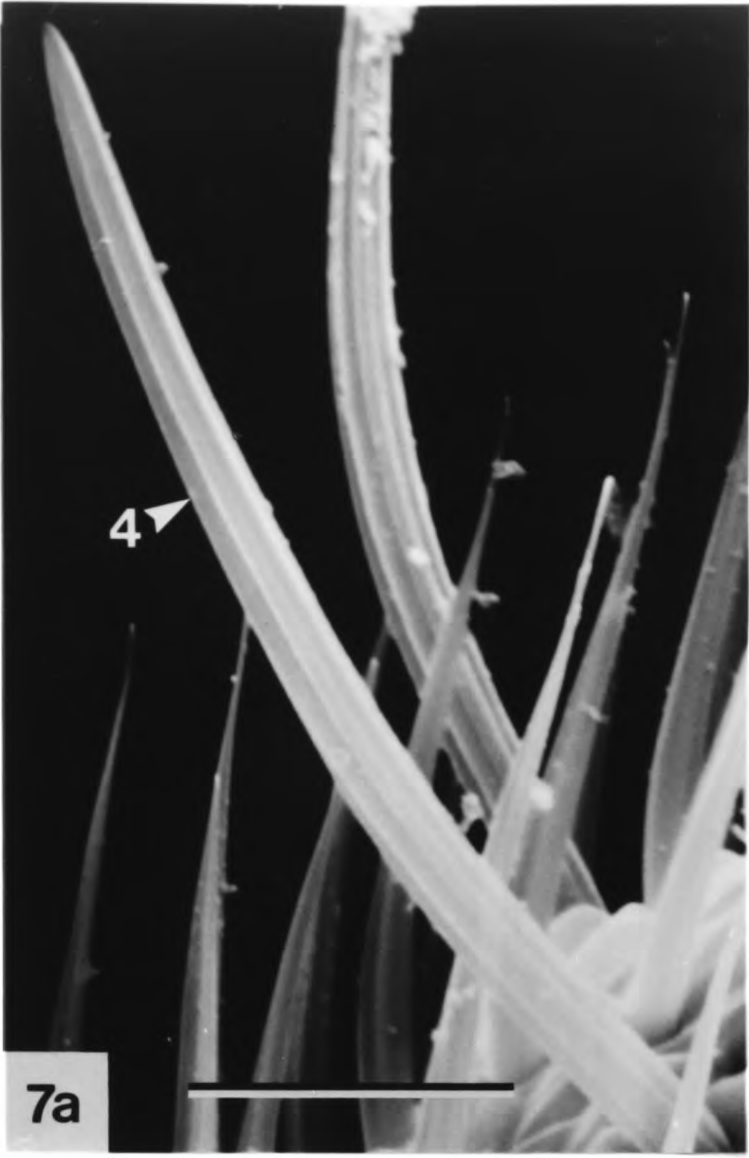


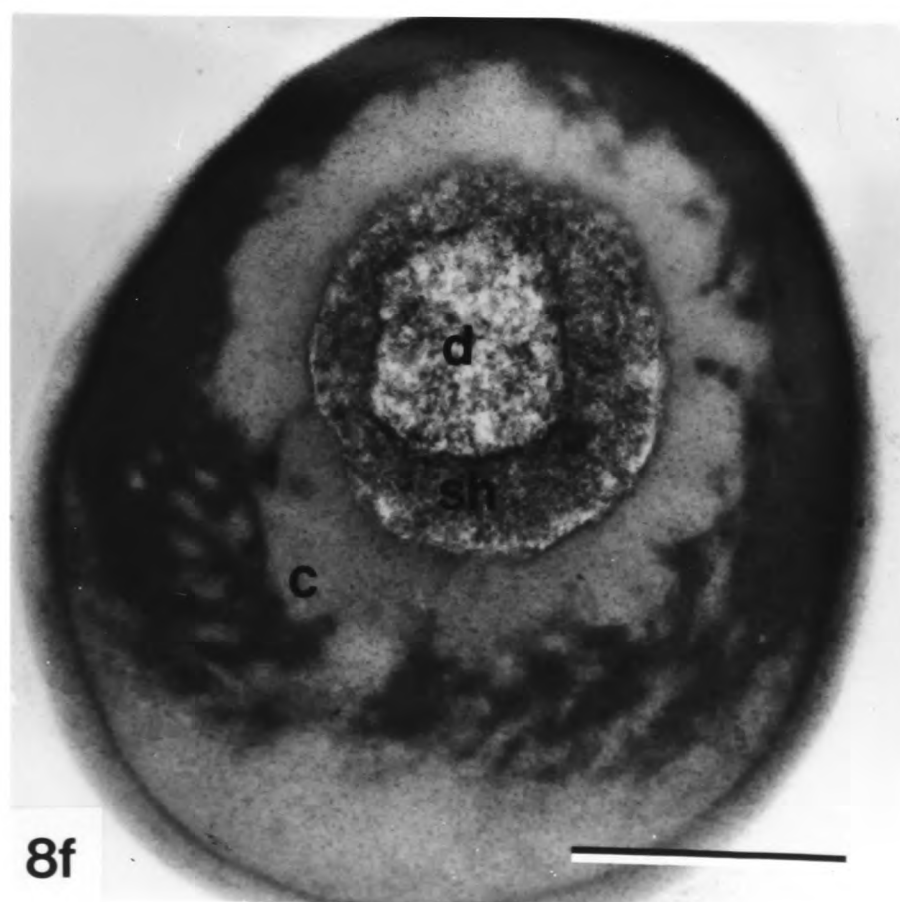
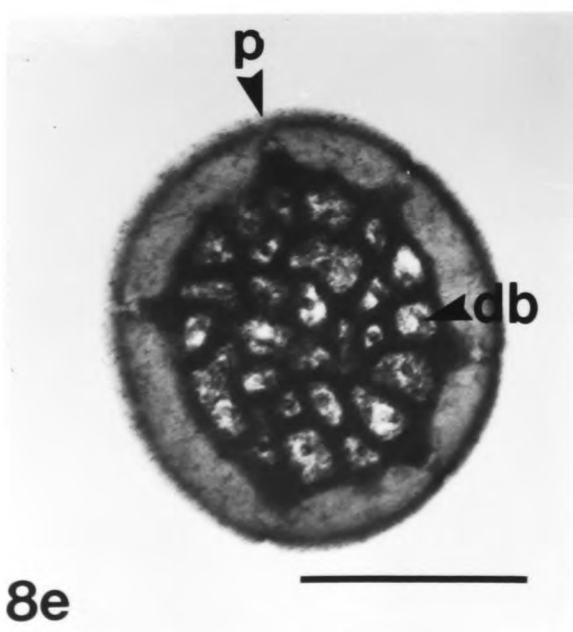
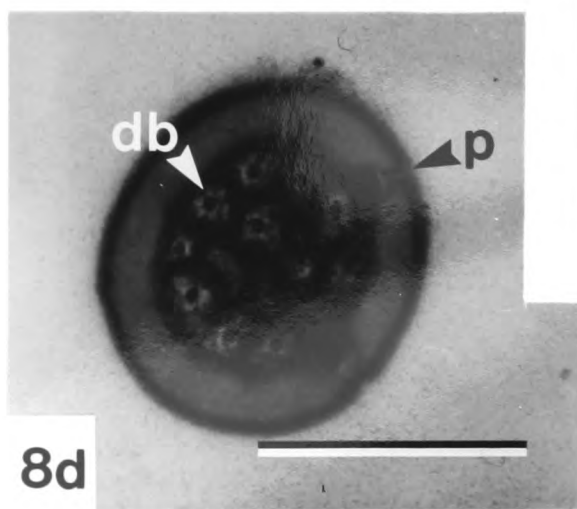
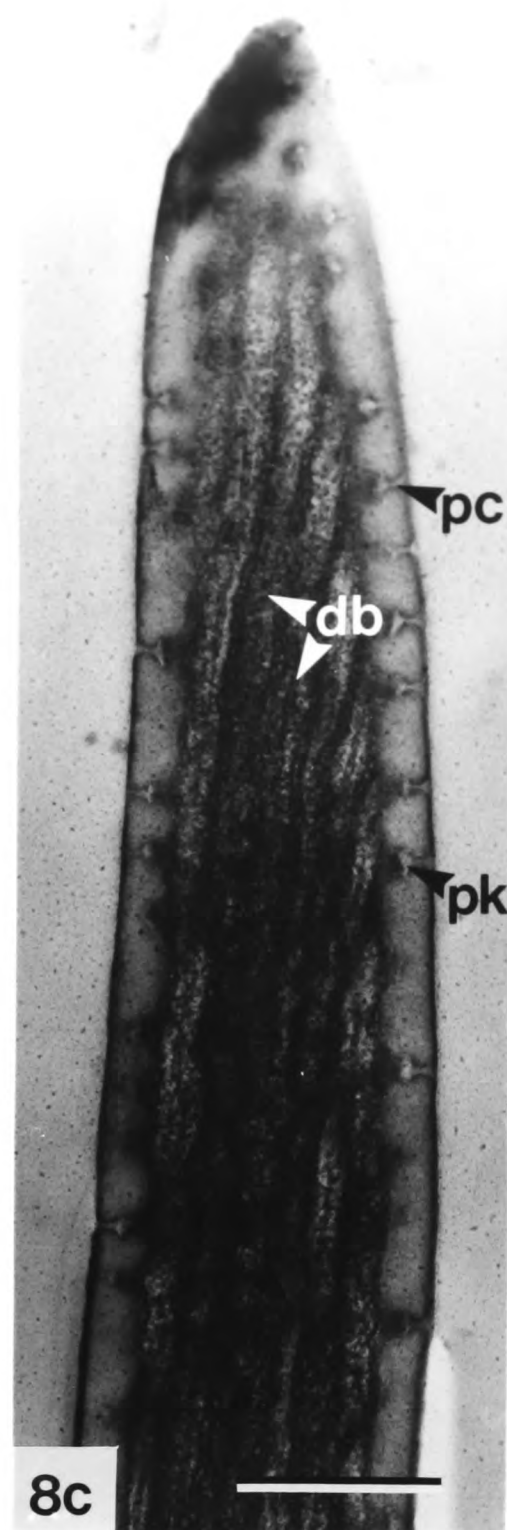
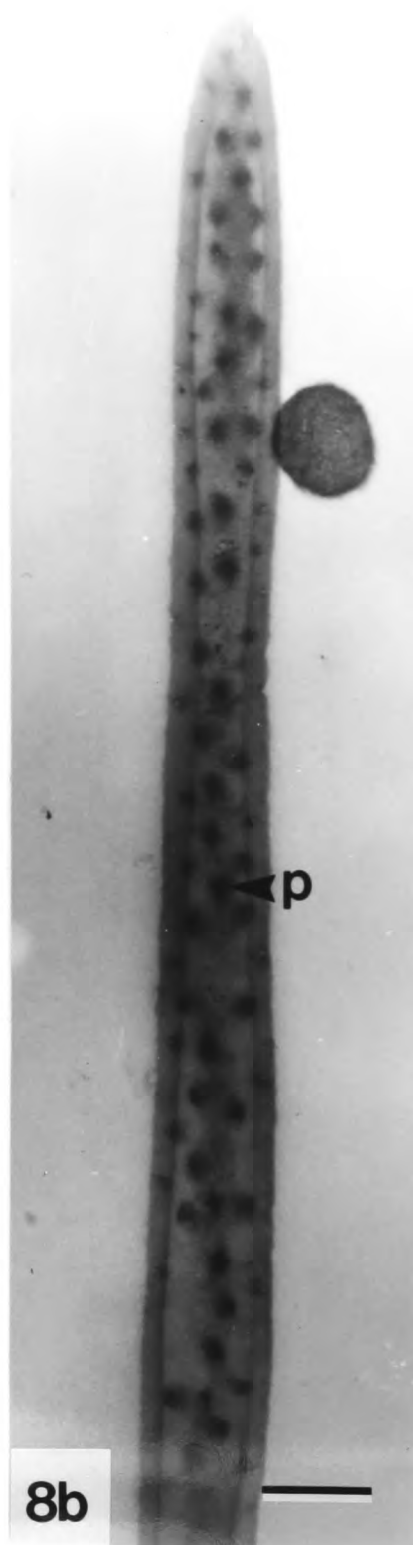
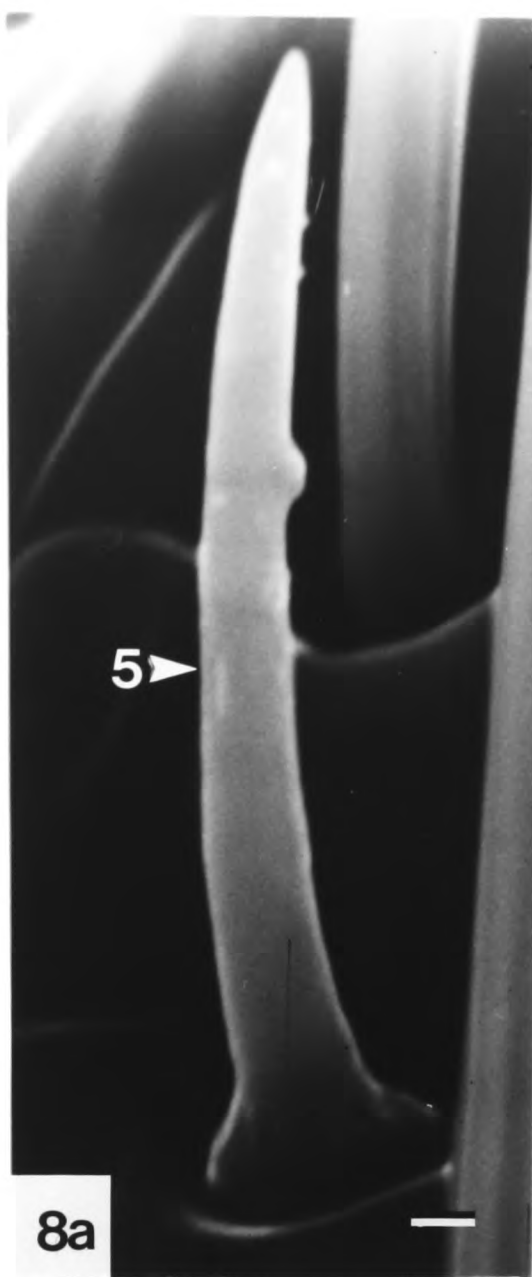


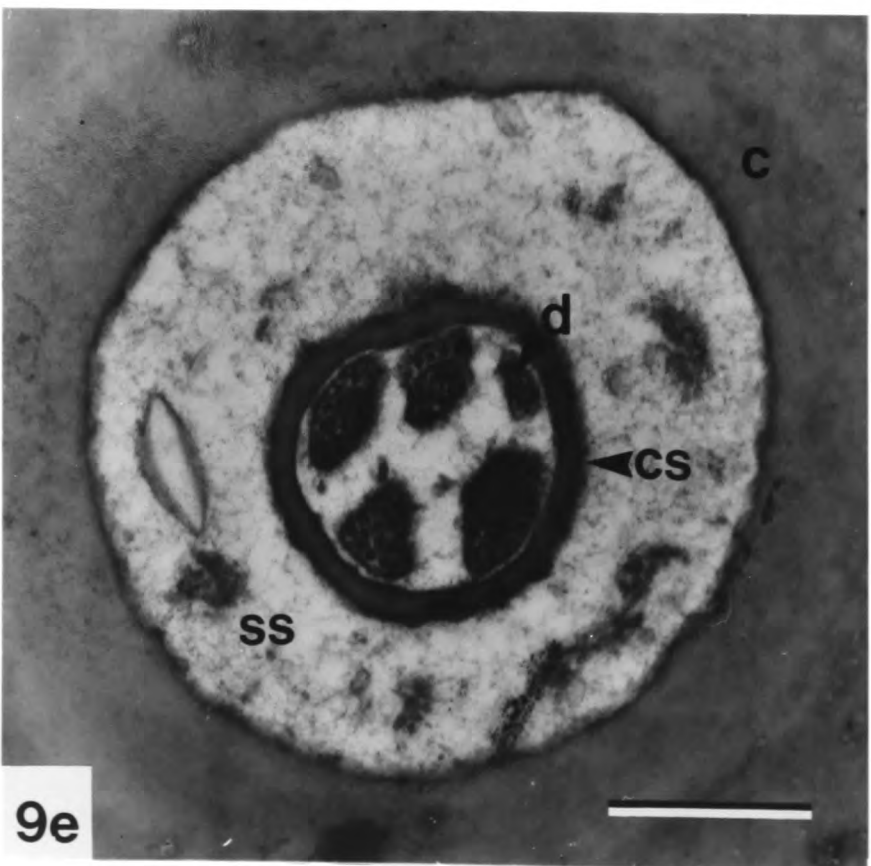
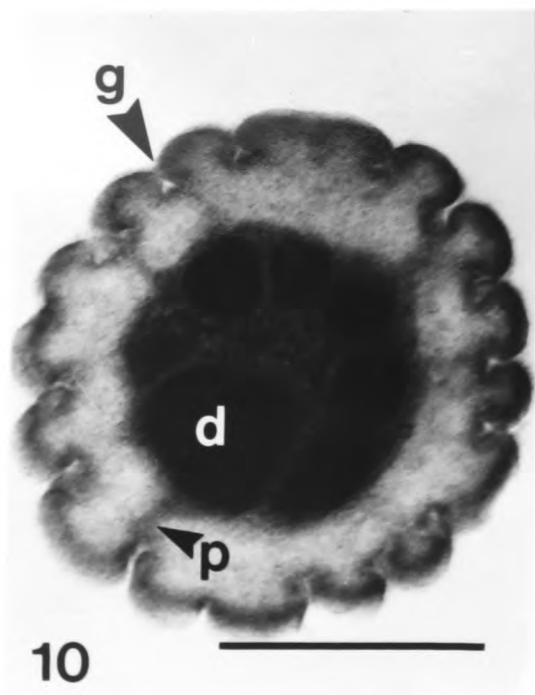
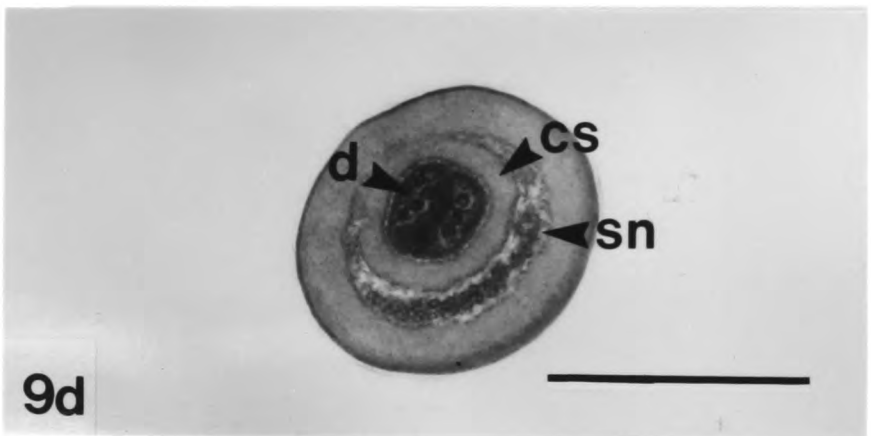
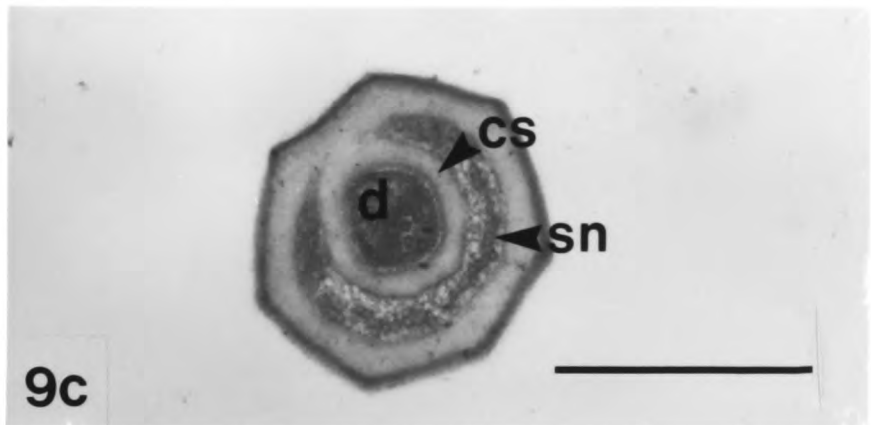
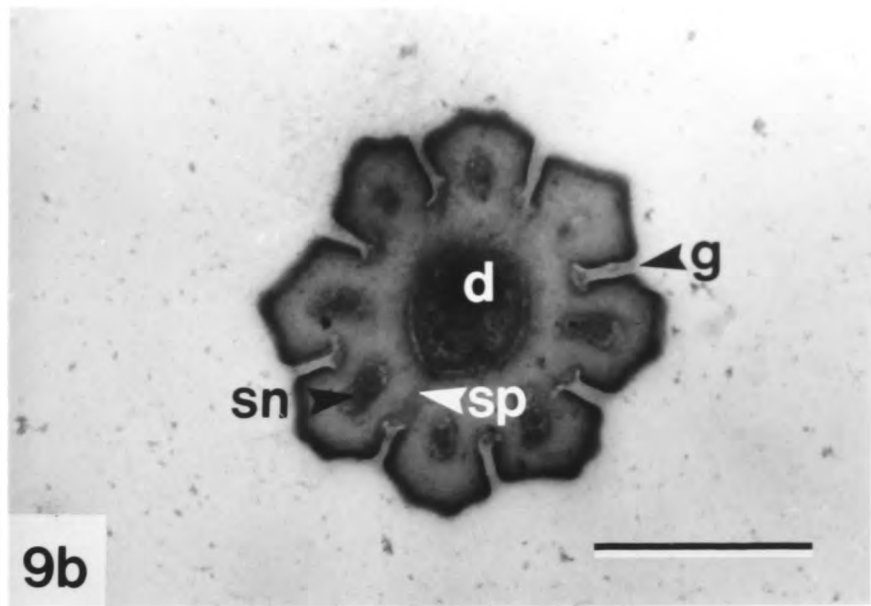
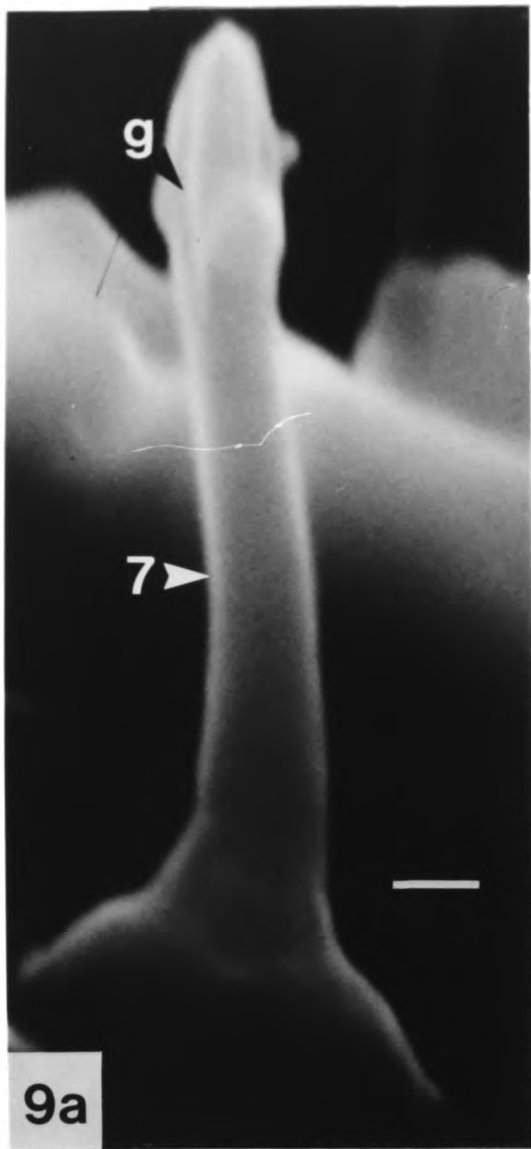




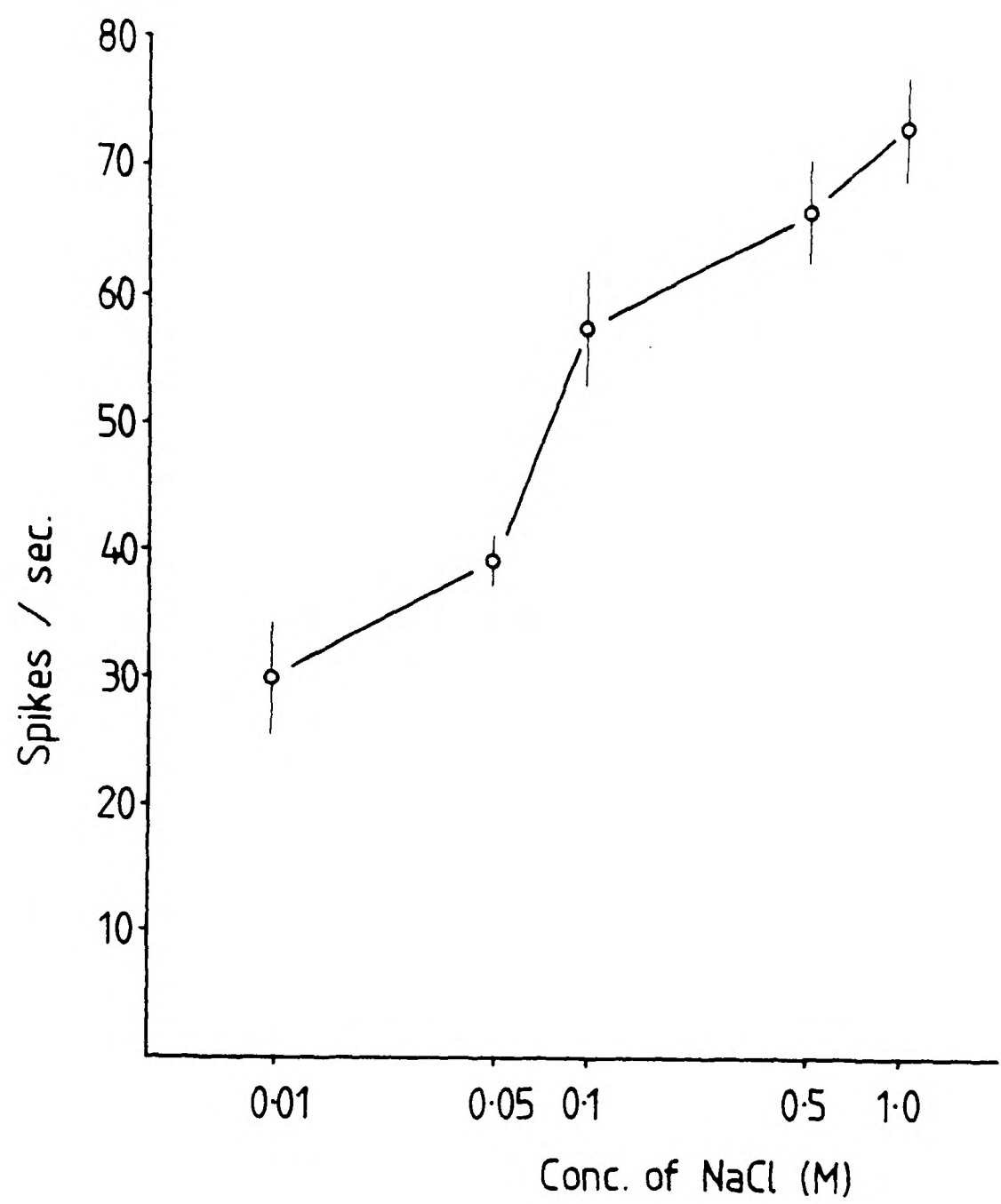








(a)



(b)

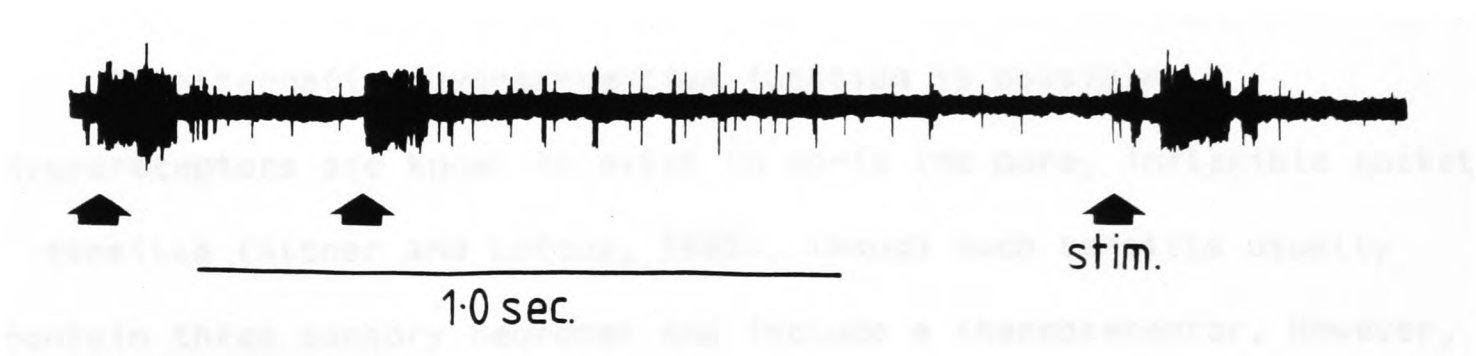


FIG.11 (a) Response of UP sensilla to Sodium Chloride concentrations.
(means $\pm$ SE, $n=12$)
(b) Response of UP sensillum to mechanical stimulation.

DISCUSSION.

Functions of sensilla types (see Table 2).

1. NPH sensilla.

NPH sensilla possess a single neurone, ending in a distinct tubular body, which suggests a mechanosensory function (McIver 1975). It is not clear from their structure whether such sensilla would be sufficiently sensitive to detect air movements as well as contact with solid objects. However, EAG studies reported below have shown that some receptors do respond to changes in the air flow over the antenna, and it is likely that NPH sensilla are involved in this response.

2. NPP-is sensilla.

The function of this type is unclear. Lack of pores, plus a single dendrite containing an ordered array of microtubules are suggestive of a mechanoreceptor. However, the dendrite tip does not constitute a distinct tubular body (cf. NPH and UP sensilla). Furthermore, mechanoreception is unlikely since these short sensilla are well shielded by longer UP and NPH sensilla.

An alternative hygrosensitive function is possible.

Hygrosensitive receptors are known to exist in np-is (no pore, inflexible socket) sensilla (Altner and Loftus, 1985), though such sensilla usually contain three sensory neurones and include a thermoreceptor. However, a conical hygrosensitive np-is sensilla with a single sensory neurone does exist on the maxillary palp of Periplaneta americana (Altner et al, 1983). The distinct, regularly arranged and often cross-linked microtubules seen in this sensillum are characteristic of such

hygroreceptive neurones.

3. NPP-fs sensilla.

These correspond closely to the np-is category reviewed by Altner and Loftus (1985). In this species the socket appears to allow for slight flexibility, though there is no evidence of a mechanoreceptive function.

Np-is sensilla have been found in small numbers, often near the tip, on every antenna where extensive searches have been made. As in this case, three sensory neurones are typically present, forming a sensory "triad" (Loftus, 1976). Triads have been studied electrophysiologically on the antennae of several insects, including P. americana (Yokohari, 1981), Locusta migratoria (Altner et al, 1981), and Carausius morosus (Tichy, 1979). In each case a combination of one cold, one moist air and one dry air receptor was found per sensillum. By comparison, the NPP-fs sensillum of O. surinamensis appears to contain two possible hygroreceptors and one possible thermoreceptor.

This sensillum type occurs singly in the pits on segments 9 and 10. In this region the electrophysiological recordings suggested the existence of a cell responding to temperature, which may have been in a sensillum of this type.

4. UP sensilla

Unbranched dendrites in the shaft of the sensillum leading to a putative terminal pore, plus the presence of a tubular body suggest a combined contact chemosensory and mechanosensory function for the UP sensilla. This was confirmed by the electrophysiological recordings.

Similar dual modality sensilla are well known in other insects (McIver 1975). Their long length allows the UP sensilla to stand clear of the other sensilla types, and their position on the three terminal segments means that they are the first part of the antenna to contact any novel object or substrate, so justifying their mechanosensory and chemosensory functions.

The UP sensillum is innervated by six neurones, of which one is concerned with mechanoreception. Of the other five, one appears to respond to salt, whilst the specificities of the other neurones have yet to be studied.. In some insects UP sensilla have also been found to respond to odours (Dethier, 1972; Stadler and Hanson, 1975) but no information is available to support this view in this species.

5,6. MPP and MPG sensilla.

MPP and MPG-sw sensilla correspond to the multiporous single-walled category of Altner (1977), and have been observed in many insects. The presence of many pores for conduction of the stimulus to the branched dendrites indicates an olfactory function for these sensilla. As in many other insects (Zacharuk, 1980), they are protected from mechanical damage by the longer NPH and UP sensilla, and by their sheltered position in pits on segments 9 and 10.

Electrophysiological recordings demonstrated the preponderance of olfactory receptors in the lateral pits of segment 10. Although it was not possible to identify the sensilla recorded, the large majority of MPP sensilla in this area suggest that the olfactory responses were from this type.

7, MPG-dw sensilla.

Various functions have been ascribed to MPG-dw sensilla in other insects. In several insects they have been shown to respond to odours (Boeckh, 1967a; Steinbrecht, 1969). The presence of pores suggests a chemosensory function, and their short length and protected position indicate a likely olfactory function in this species. There thus appear to be three structurally distinct types of olfactory receptors. In Periplaneta americana, Altner et al (1977) found that MPP (mp-sw) and MPG-dw (mp-dw) sensilla have different specificities:- MPP sensilla responding to short chain alcohols whilst MPG-dw sensilla respond to short chain acids and amines. Further work will determine whether the different receptor types function in a similar way in O. surinamensis.

Dual modality of MPG-dw sensilla is also possible, since temperature receptor units have also been recorded from similar structures (Altner and Loftus, 1985). In Periplaneta americana (Altner, 1977) and Locusta migratoria (Altner et al, 1981) cold receptors occur in the same MPG sensilla as olfactory receptors for hexanoic acid. Such dual modality sensilla were indistinguishable morphologically from purely olfactory MPG sensilla, so it is not possible to deduce a temperature receptor function in this case on purely structural features.

Sensilla numbers.

(i) mechanoreceptors.

Over 68% of the antennal sensilla of O. surinamensis are concerned with mechanoreception, suggesting the importance of tactile

stimuli. Both in its original habitat beneath bark, and within bulks of stored products, these beetles inhabit small crevices where contact with the substrate will be considerable. In the laboratory, it has been shown that O. surinamensis maintains a high level of contact stimulation whenever possible (Arbogast and Carthon, 1973), and this is likely to be mediated by mechanoreceptors on the antennae, legs and body surface.

(ii) Chemoreceptors.

Chemoreceptors constitute less than 30% of antennal sensilla, with a mean total of 220 (29 contact/191 olfactory). Compared to studies of other coleopterans, (see Chapman, 1982) this figure is very low. However, this species has been shown to respond behaviourally to a variety of olfactory stimuli (Pierce et al ., 1981; Stubbs et al ., 1985).

In his extensive review, Chapman (1982) recognised several selection pressures likely to affect numbers of chemoreceptors, including insect size, sex, feeding habits and the need for sensitivity. Numbers of sensilla correlate well with antennal length in endopterygotes. With an antennal length of 0.8mm, O. surinamensis falls within the predicted range of sensilla numbers. The low numbers may therefore simply result from small body size.

Numbers of olfactory sensilla are also likely to reflect feeding habits, in particular the extent to which olfaction is used to locate food resources, and the abundance and distribution of suitable food. Comparisons with other stored product beetles would be instructive, but information is only available for Tenebrio molitor (Harbach and

Larsen, 1977). This species has considerably more olfactory and contact chemoreceptors, but again this can be accounted for by its larger size.

Low numbers of olfactory sensilla suggest a low level of sensitivity to odours, which in turn suggests that odours are not important in the location of conspecifics or food sources over large distances. This will be discussed further after consideration of the sensitivity of both antennal and behavioural responses to such odours.

CHAPTER 3.

THE ELECTROANTENNOGRAM RESPONSE - A CRITICAL STUDY.

INTRODUCTION.

Since its introduction by Schneider (1957), the electroantennogram (EAG) has been used extensively as a "rapid and convenient screening procedure for pheromones" (Roelofs, 1984). The technique has been successfully used to identify pheromone components in the Lepidoptera (Roelofs, 1984) and in other orders including Coleoptera (Payne, 1979; Levinson *et al* , 1978; Wadhams, 1984), Orthoptera (Nishino and Kimura, 1981,1982; Nishino and Takayanagi, 1981) and Hymenoptera (Kraemer *et al* , 1981; Williams *et al* , 1982). In contrast, however, little attention has been paid to the generation of the EAG response and its relationship to the numbers of olfactory receptors stimulated.

The EAG response is generally accepted to be "essentially the sum of many olfactory receptor potentials recorded more or less simultaneously by an electrode located in the sensory epithelium" (Schneider, 1963), but little evidence has been presented to show how these receptor potentials combine to give the EAG response. Previous studies of the nature of the EAG response fall into 2 categories. The first contains investigations of the effect of varying the proportion of the antenna - and hence the numbers of sensilla - stimulated (Nishino and Takayanagi, 1979; Roelofs and Comeau, 1971; Mayer *et al* , 1984). In only one of these studies have the actual numbers and

distribution of sensilla been determined (Mayer et al , 1984). The other category contains investigations of the variation in the EAG response recorded along the length of an intact antenna, by altering the position of the recording electrode (Payne et al , 1970; Behan and Schoonhoven, 1978; Nagai, 1981). In each of these cases, the EAG response has been found to vary along the length of the antenna, but this may have been due to at least two different factors: the number of sensilla included between the recording and indifferent electrodes (Nagai, 1981), and the actual distance between the electrodes. These experiments have been conducted only on Lepidopteran antennae, where the olfactory sensilla are distributed over the entire antennal surface, and so these two effects could not be separated. In O. surinamensis , however, the antenna is differentiated into regions with (segments 9-11) or without (segments 1-8) olfactory sensilla (White and Luke, 1986; Chapter 2). It is therefore possible to determine how the EAG response varies in these two regions.

Three EAG experiments were therefore conducted to examine critically the EAG response in O. surinamensis :-

i. The EAG response to Carob Distillate.

Males and females were presented with a range of concentrations of this food odour to look for any sexual differences in their responses.

ii. The effect of sensilla numbers on EAG amplitude.

Since the numbers and distribution of each sensillum type on the antenna of O. surinamensis have been elucidated (Chapter 2), the

numbers of sensilla were varied by segment removal to investigate the effect on the amplitude of the EAG response to both the food odour and the blank response.

iii. The effect of recording electrode position on EAG amplitude.

This was conducted to examine how the EAG response varied along the intact antenna. Previous work (Nagai, 1981) suggests that there should be a response gradient along regions bearing olfactory sensilla (i.e. an increase from segments 9-11), but no previous recordings have been reported from regions where olfactory sensilla are absent (segments 1-8). This experiment should therefore contribute to our understanding of the EAG response.

MATERIALS AND METHODS.

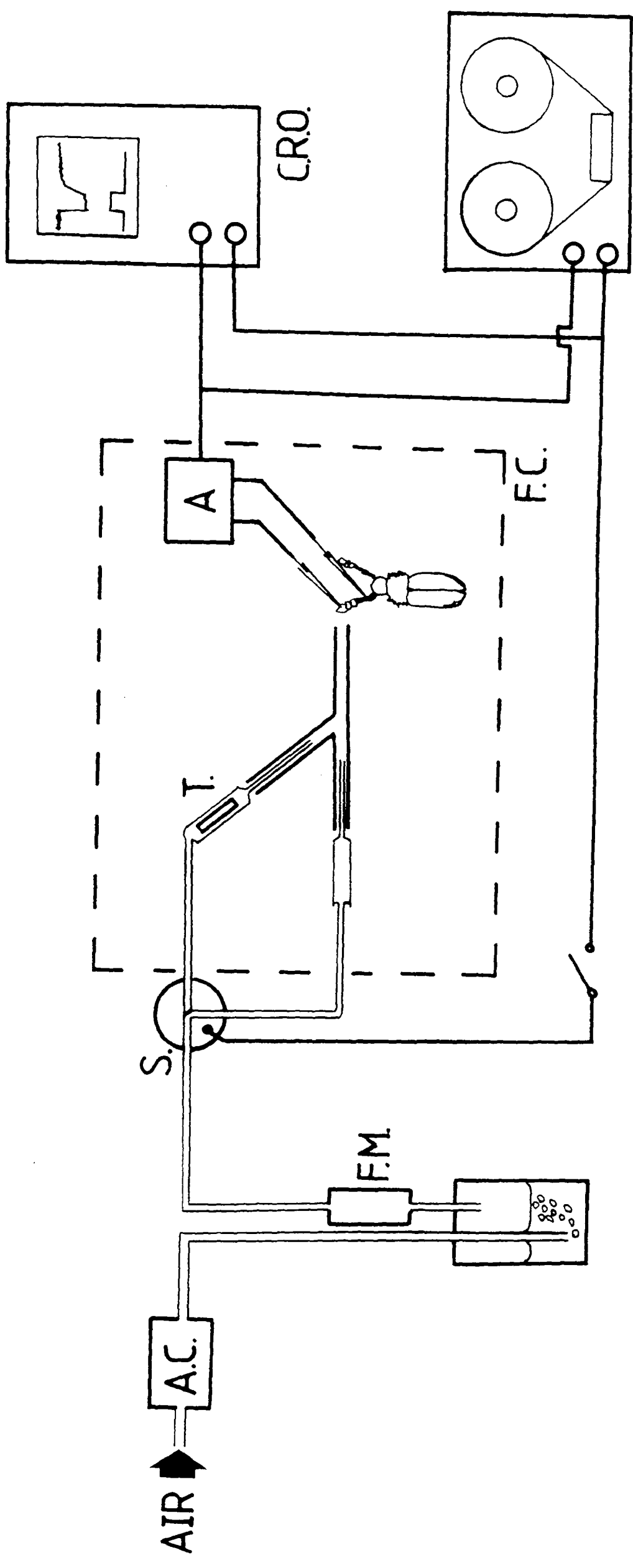
Insect material.

Cultures of O. surinamensis were kept as described in Chapter 2. Beetles used in this study had eclosed to become adults 2-4 weeks previously, and were removed from the culture jars and kept in clean glass 5 cm x 2.5 cm tubes for 24 h before use.

Electroantennogram (EAG) recording system.

Electroantennograms were recorded from intact individuals mounted ventral side uppermost on a cork stage, using double-sided adhesive tape. Glass electrodes (drawn from 2mm o.d. capillary tubes) filled with saline (Roelofs, 1984) were connected, using chloridised silver wires, to a Grass P-16 DC preamplifier used in differential mode (Fig. 12). Resulting traces were displayed on a Tektronix 5223 storage oscilloscope, and recorded using a Racal Store 4DS DC tape recorder. Permanent traces were produced on a Medelec UV recorder.

Due to the toughness of the cuticle, glass capillary electrodes could not be used to penetrate the antenna. Consequently, small holes were punched in the cuticle using a finely etched tungsten needle, prior to insertion of the electrodes. Under 100x binocular magnification, the recording electrode was inserted into the terminal (11th) antennal segment. In preliminary recordings, the indifferent electrode was inserted through the soft intersegmental membrane between the head and the thorax, but this resulted in unacceptable levels of electrical noise. To overcome this, the electrode was inserted into a small hole punched in the 2nd segment of the antenna.



ELECTROANTENNOGRAM (EAG) RECORDING SYSTEM

FIG.12. Electroantennogram recording system. (A-amplifier, A.C.-activated charcoal, C.R.O.-cathode ray oscilloscope, F.C.-Faraday cage, F.M.-flowmeter, S-solenoid valve, T-test cartridge)

Set up in this manner, preparations gave reproducible EAG responses, with little background noise, for up to 5 hours.

The odour delivery system used bottled compressed air, which was filtered through activated charcoal, humidified, regulated at 1000 ml/min using a flowmeter, and then passed to a two-way solenoid valve, controlled by a timer giving a one second pulse (Fig. 12). When inactivated, the solenoid directed the air via the clean air line:- teflon tubing leading to an empty pasteur pipette inserted into one arm of a glass "Y" delivery tube. When activated, the solenoid diverted the air via the test cartridge line:- an equal length of teflon tubing connected to the test cartridge, which was inserted into the other arm of the "Y" delivery tube. The outlet of the delivery tube was aligned along the antennal axis, at a distance of approximately 1 cm, so that the whole antenna was within the airstream. To ensure that odours were removed after presentation, an exhaust system was suspended over the preparation. This consisted of a 10cm diameter perspex cylinder connected by flexible piping to a fume hood. Air was continually drawn from around the preparation into the fume hood, and then expelled from the room. The extractor fan was kept permanently on, maintaining a constant airflow through the room between and during experiments, and preventing the accumulation of contaminating odours.

Test cartridges consisted of a 4cm x 4mm filter paper strip impregnated with the test material, inside a pasteur pipette. Recordings were made between 0900 and 1800 hrs, at room temperature (22°- 24°C).

Olfactory stimuli.

An aqueous distillate of carob pods (Ceratonia siligua L.) was used as an olfactory stimulus in these experiments. The preparation of carob distillate (CD) has been described previously (Stubbs et al , 1985), and the chemistry of this mixture is discussed in Chapter 4. Carob distillate was used because initial tests recorded EAG responses of sufficient amplitude to allow variation with numbers of receptors stimulated, and with electrode position, to be investigated.

Experimental Procedure.

i. EAG responses of O. surinamensis to carob distillate.

Preparations were set up as described above and left for 30 mins for the baseline to stabilise before recordings were made. Each preparation was then exposed to a series of cartridges containing 10ul of various dilutions of CD, in ascending order of concentration. Prior to each test cartridge, a water blank was used, consisting of 10ul of distilled water applied to a filter paper strip. Test presentations were separated by 5 mins to allow the receptors to recover fully between stimuli. The EAG amplitude was measured from the stored trace as the maximum DC deflection from baseline (Fig. 13). The response to the water blank represented the response to water, contaminating odours and the mechanical disturbance in the airflow. This response was subtracted from the test response to give the net EAG response to CD, which was used in the analysis.

Recordings were made from 20 individuals (10 male, 10 female) and results analysed by a 2 level nested analysis of variance (Sokal and Rohlf, 1969) to determine any sexual differences in the responses.

ii. The effect of sensilla numbers on EAG amplitude.

Preparations were set up as described above, but with the recording electrode inserted into segment 8 i.e. 4 segments from the tip. A standard stimulus of 10ul of CD was presented, plus a water blank and a dry blank (dry filter paper strip in a pasteur pipette) and the EAG responses recorded. This series was then repeated, and the results averaged. The electrodes were then withdrawn, the terminal segment carefully removed using the tip of a scalpel blade, and then the electrodes re-inserted in their original positions. After 10 mins the above series of presentations was repeated. In this way the antenna was tested after progressive removal of segments 11, 10 and 9. A total of 10 individuals were used, 5 male and 5 female.

Results were analysed by regression analysis (Sokal and Rohlf, 1969) to investigate the effect of segment removal on the EAG response to CD (response to CD - response to water blank), water (water blank - dry blank) and the response to the dry blank.

iii. The effect of recording electrode position on EAG amplitude.

After individuals were mounted as outlined above, a small hole was punched in the cuticle of each antennal segments 2 - 11, to allow for electrode insertion. The indifferent electrode was inserted into segment 2 as before, the recording electrode into segment 11, and a standard stimulus of 10ul of CD was presented. This was repeated with the recording electrode placed sequentially in each segment of the antenna. After every 2 test presentations the recording electrode was returned to segment 11 and the response remeasured. By using this EAG response as a standard, and expressing all results as a % of the mean of the standard responses before and after each test, it was possible

to control for the effects of individual variation in sensitivity, and any deterioration of the preparation, in the subsequent analysis.

A total of 20 individuals were used (10 male and 10 female), although it was not possible to record from all segments in every preparation. Regression analysis was used to investigate the effect of recording electrode position on EAG amplitude. Separate analyses were conducted for the antennal segments bearing olfactory sensilla, and for those without, and the slopes of the regression lines in the two regions compared.

RESULTS.

i. EAG response of O. surinamensis to carob distillate.

O. surinamensis gave a typical EAG response to CD (Fig. 13), with a sharp depolarisation, followed by a slower recovery phase. The effect of CD concentration on EAG amplitude (Fig. 14) showed a threshold for response at approximately 1/100 dilution, and that the response continued to increase up to the highest concentration tested. The responses of males and females were not significantly different ($P > 0.05$).

ii. Effect of sensilla numbers on EAG amplitude.

Progressive removal of the terminal segments caused successive reduction of the EAG amplitude, reaching zero after the removal of the three club segments (Fig. 15a). Regression analysis of EAG amplitude v number of club segments remaining gave a highly significant linear relationship ($F=77.1$, $r=0.811$ $P < 0.001$; slope $b=142.6$ uV/segment). Using data from Chapter 2, regression analysis of EAG amplitude v number of olfactory sensilla (sensilla types MPP + MPG-sw + MPG-dw) also gave a highly significant fit to a straight line ($F=53.7$, $r=0.757$ $P < 0.001$; slope $b=1.45$ uV/sensillum), although the correlation was lower than with segment number (Fig. 15b).

Analyses of EAG responses to water vapour (water blank - dry blank) and to the dry blank (Fig. 15a) both gave positive linear relationships to the number of club segments remaining (water vapour: $F=6.51$, $r=0.374$ $P < 0.05$, slope $b=7.38$ uV/segment; dry blank: $F=44.8$, $r=0.727$ $P < 0.001$, slope $b=30.61$ uV/segment).

A possible criticism of this experiment is that the decrease

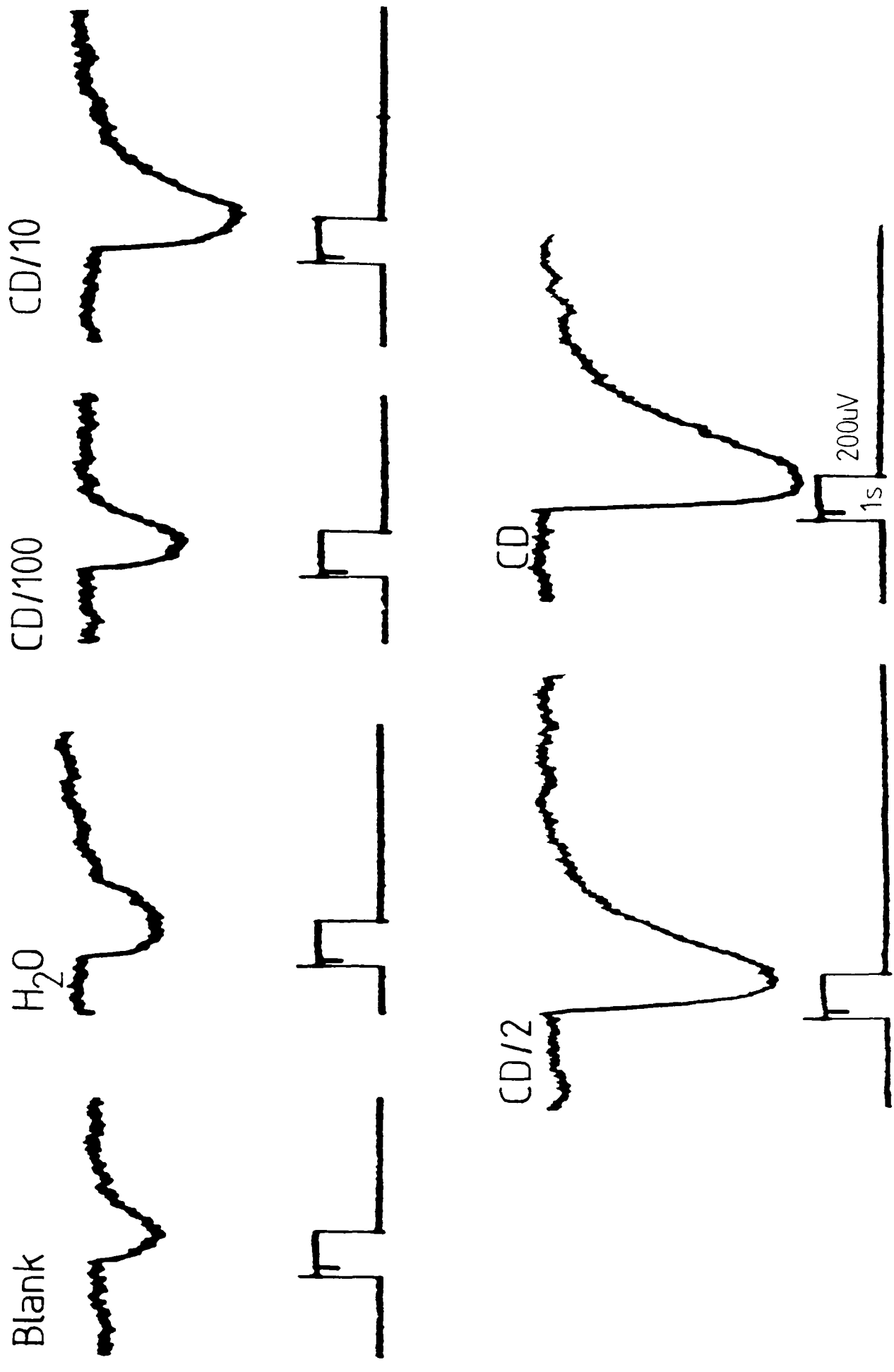


FIG.13 EAG response of O. surinamensis to Carob Distillate. Sample traces from one insect.

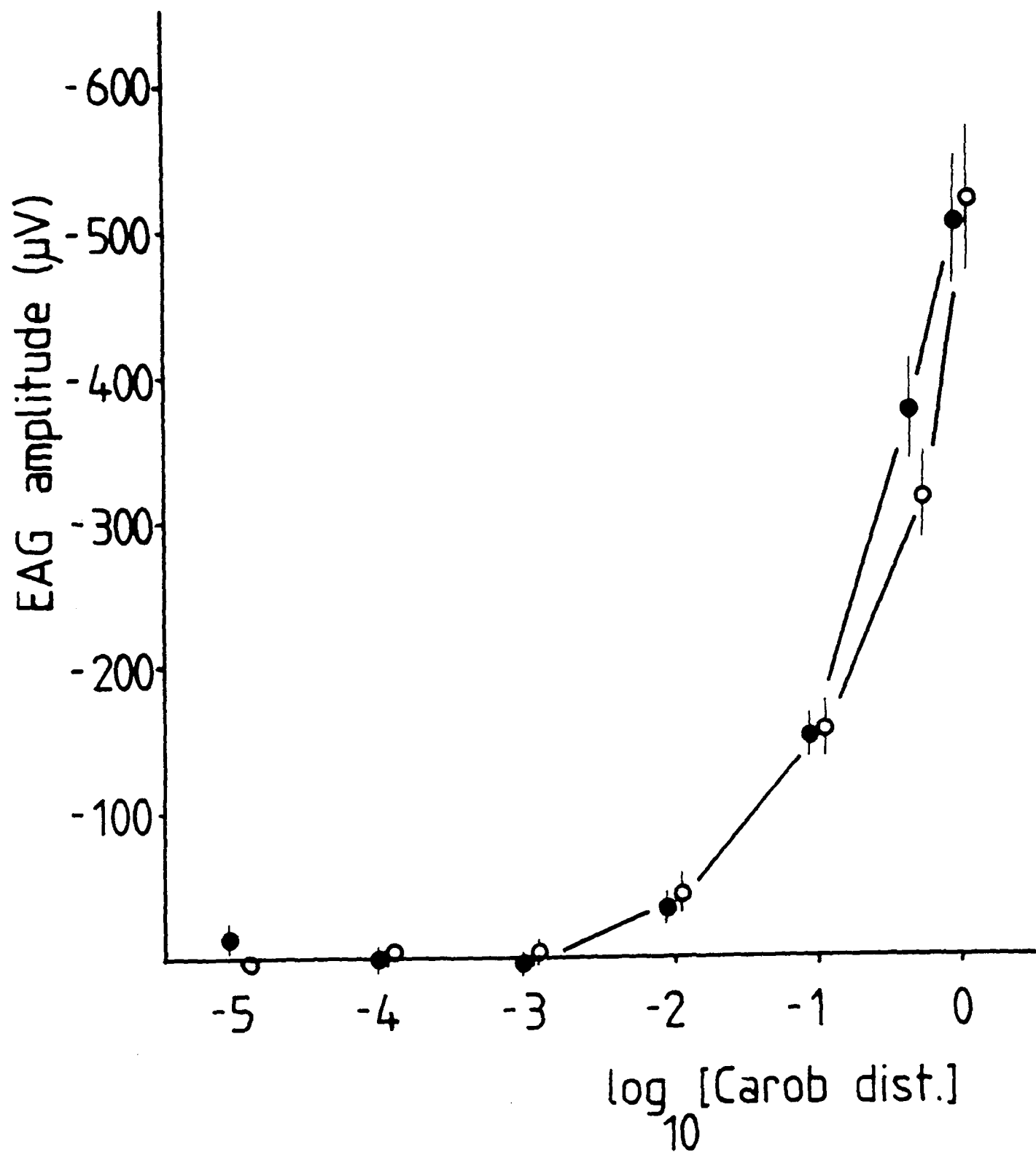


FIG.14. EAG response of Males (○) and Females (●) to Carob Distillate.
(means $\pm$ SE, n=10)

observed after sectioning the antenna was due to deterioration of the preparation with time. The procedure prevented the use of a standardisation method as used below. However, to test for deterioration, one preparation was repeatedly tested over a period of 30 mins after one segment had been removed and the response remained steady throughout this period (340uV at 0 min, 300uV at 10 min, 350uV at 20 min, 330uV at 30 min).

iii. Effect of recording electrode position on EAG amplitude.

Results from segments 3-8 (Fig. 16) showed it was possible to record a normal EAG response from segments that do not themselves carry olfactory receptors. Analysis of the EAG responses to the standard CD stimulus from each of the segments of the intact antenna (Fig. 17a) demonstrated two functionally different regions which correlate with the distribution of olfactory sensilla. From segment 2 to 9 (sensilla absent), the EAG amplitude rose linearly along the antenna (slope $b=17.3 \pm 2.0$ %/segment) whilst, unexpectedly, from segment 9 to 11 (sensilla present) there was a small but insignificant decrease (slope $b=-3.6 \pm 3.8$ %/segment). The slopes for the 2 sections of the antenna were highly significantly different ($t=4.82$, $P<0.001$).

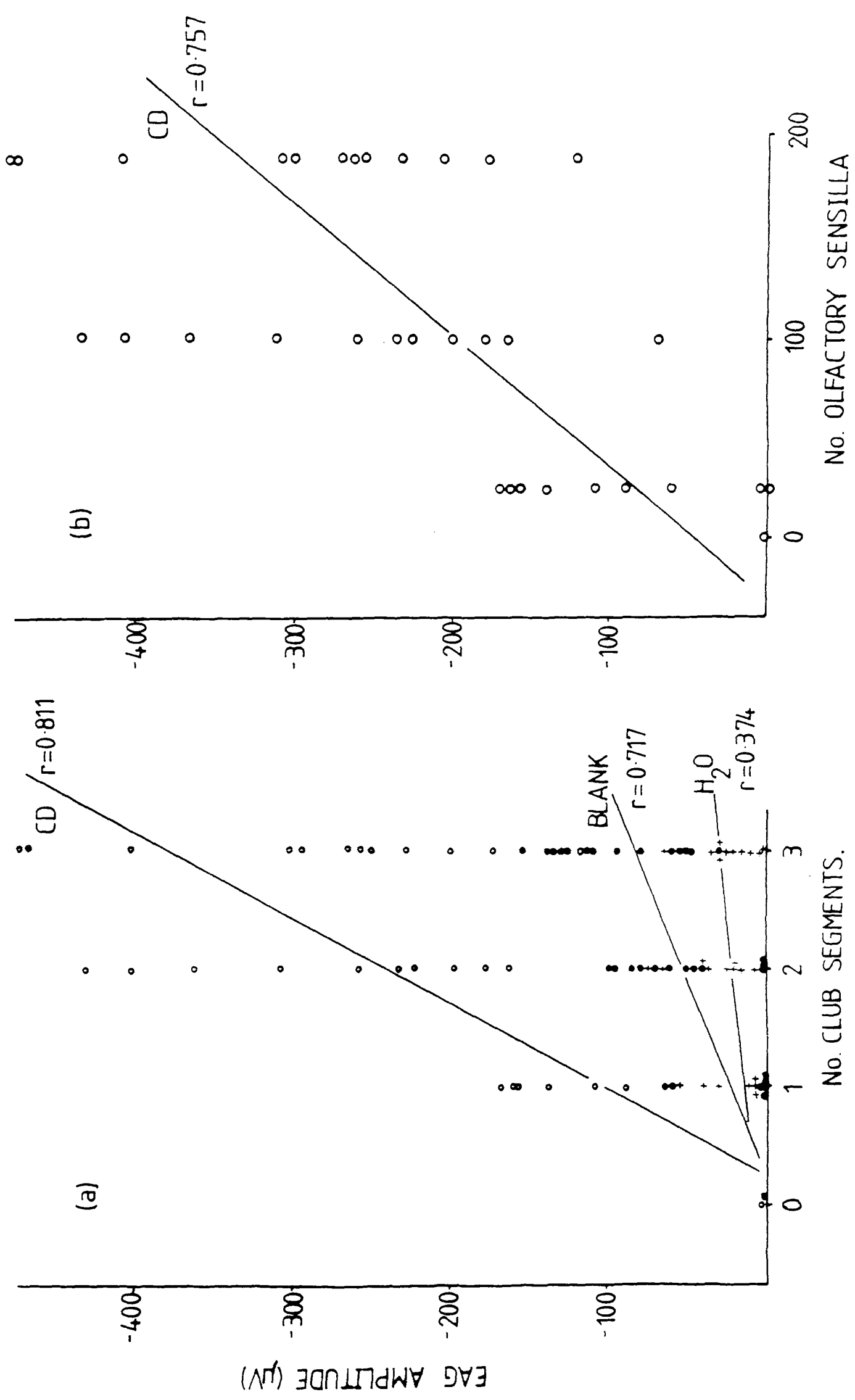


FIG.15 (a) EAG responses recorded to Carob Distillate (CD), water vapour and blank cartridge versus number of club segments present on antenna. (CD=○, water=◐, blank=+). (n=12 individuals)

(b) EAG response recorded to Carob Distillate (CD) versus number of olfactory sensilla on antenna. (n=12 individuals).

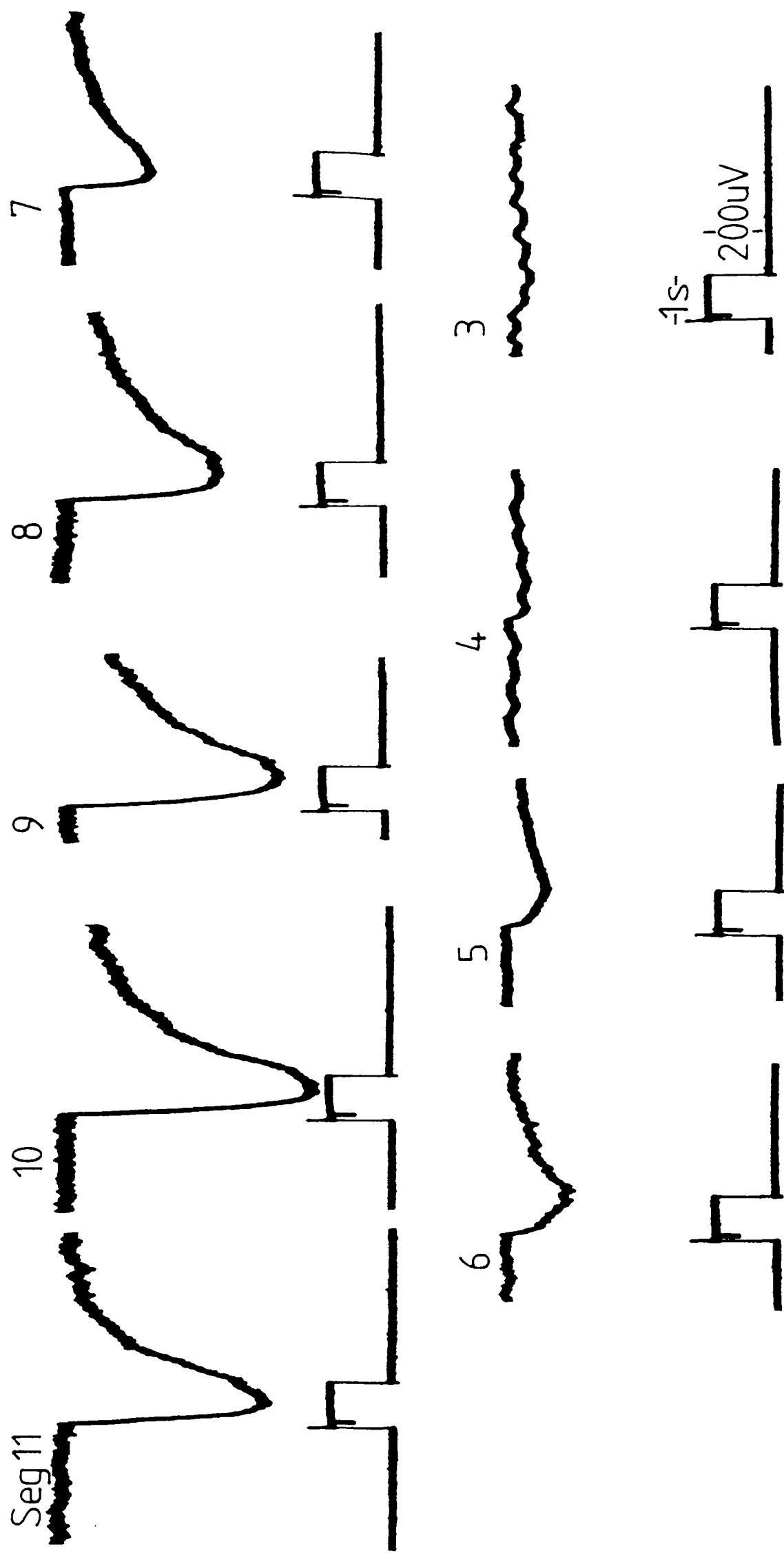


FIG.16 EAG response to Carob Distillate (10ul) recorded from each segment of an intact antenna.
Indifferent electrode positioned in segment 2.

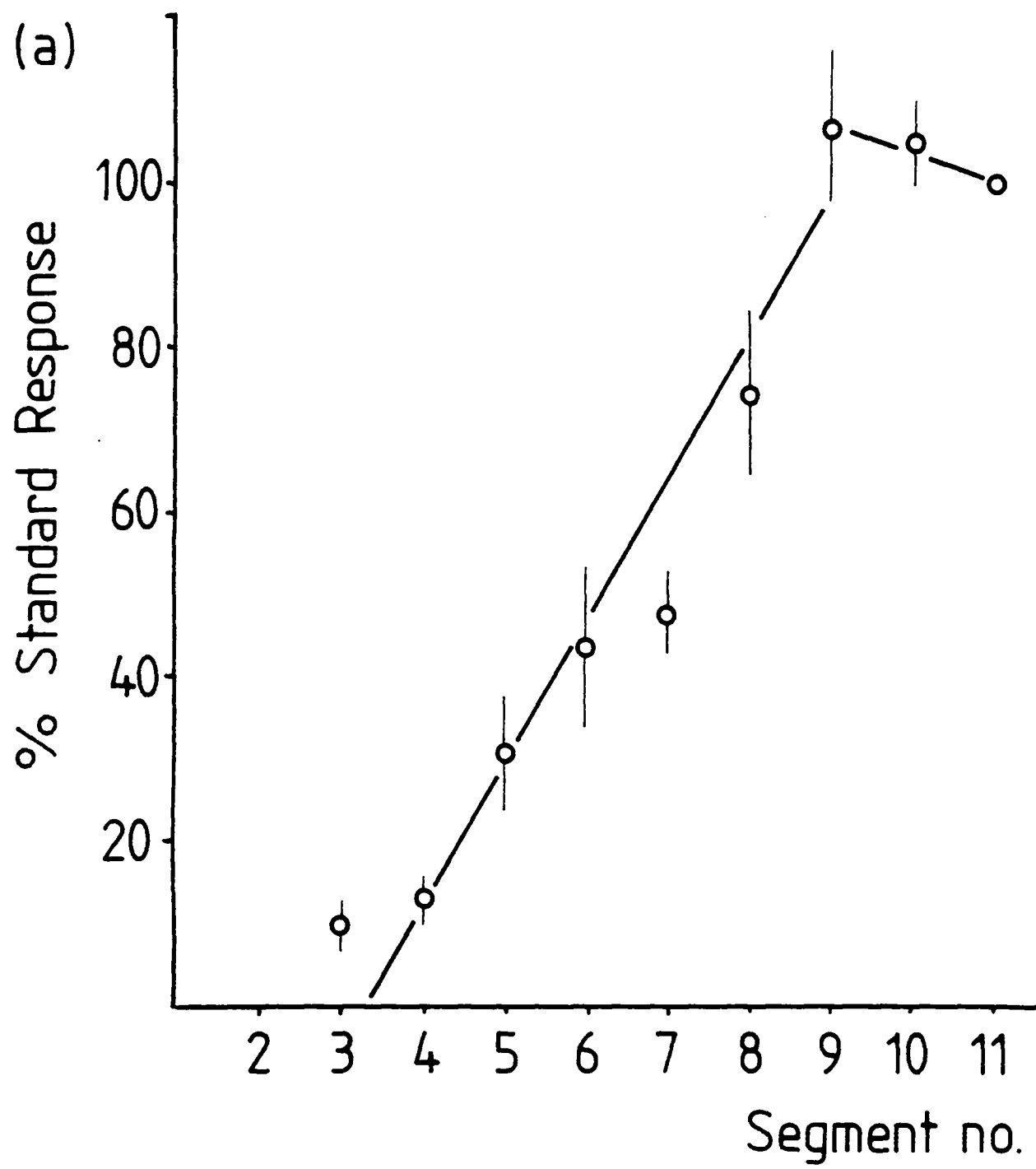


FIG.17 (a) EAG response to Carob Distillate recorded from each segment along an intact antenna. (Calculated as a % of the response recorded from the terminal segment. Means $\pm$ SE, $n=20$)

(b)

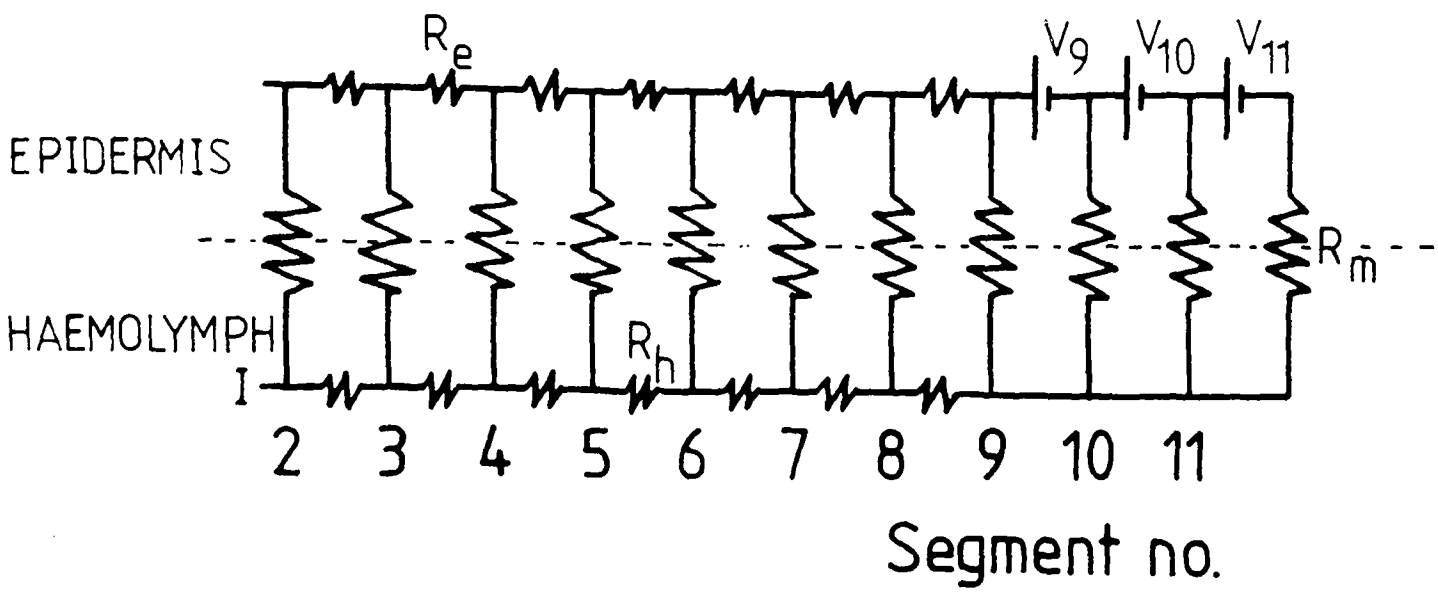


FIG.17 (b) Simple electrical model of antenna.
 R_e = Resistance of epidermal layer between segments.
 R_h = " " haemolymph channel " "
 R_m = " across basement membrane in each sement.
 V_9 = Sum of receptor potentials generated in segment 9.
 V_{10} = " " " " " " " 10.
 V_{11} = " " " " " " " 11.
I = Position of indifferent electrode.

DISCUSSION.

The EAG responses of the intact antenna to CD (expt.i) agree with the structural evidence showing no differences in sensilla number or distribution between males and females (Chapter 2). The lack of difference in EAG response between the sexes also suggests that there are equal numbers of receptors for this food odour.

The effect of segment removal was also compatible with the structural evidence. The decrease of the response, eventually to zero, with the removal of segments 11, 10 and 9 showed that there were olfactory receptors for CD on these 3 segments, but not on the rest of the antenna. This matches the distribution of olfactory sensilla.

The EAG amplitude was shown to be directly proportional to the number of club segments and also the number of olfactory sensilla on the antenna. The better fit to the former may reflect the difficulty in determining the actual number of sensilla responding as their specificities are not known. Another possible contributing factor was aspect, since all sensilla were not equally exposed to the stimulating odours. Although the terminal segment possessed the greatest number of olfactory sensilla, many of them were occluded by the mounting procedure i.e. sandwiched between the antenna and the adhesive tape. This probably explains why the EAG response did not decrease as much as expected after the removal of segment 11. Due to their position on the distal surfaces, the sensilla on segments 9 and 10 were not occluded by the mounting procedure, which may explain the larger decrease in the EAG amplitude after the removal of these segments. Despite these complications, however, the linear relationship between the EAG amplitude and the number of olfactory sensilla stimulated

suggests that the potentials generated by individual receptors are summed to give the overall EAG response, and therefore, that such potentials are connected electrically in series.

Roelofs and Comeau (1971) obtained a similar result in the red-banded leaf roller, Argyrotaenia velutinana. Progressive sectioning of the antenna showed that the EAG amplitude was directly proportional to the length of antenna remaining, although sensilla numbers were not determined. In the only previous study with known numbers of sensilla (Mayer et al, 1984), the EAG response of Trichoplusia ni to sex pheromone was shown to be directly proportional to the number of sensilla left on the antenna after each section. Furthermore, in the same study, stimulation of small numbers of sensilla by confinement of the stimulus to parts of the antenna showed the same relationship.

Segment removal also provided clues as to the nature of the "blank" response. In EAG studies these are usually subtracted from the test response without determining their origin. The dry blank response in the present study was directly proportional to the number of club segments left on the antenna, and fell to zero after the removal of all 3 terminal segments, reflecting the distribution of the olfactory receptors. In their experiment, Mayer et al (1984) concluded that the blank response was due to at least 3 different stimuli:- water vapour, extraneous room contaminants and plasticiser from the delivery line. Clearly, the nature of the blank response is likely to vary between different species, depending on the types and numbers of sensilla present, and between different presentation techniques. In

the present case, it is not possible to exclude the contribution of mechanoreceptors (the most numerous sensilla type -see Chapter 2) responding to disturbance in the air flow during stimulation, since repeated purging of the delivery line (to remove odour contaminants) reduced the blank response amplitude, but not to zero. The blank response was therefore probably due to a combination of mechanical disturbance and contaminating odours.

The water response recorded was small, but was consistent with the distribution of NPP-is and NPP-fs sensilla (suggested hygrometers - see Chapter 2).

Altering the recording electrode position showed a linear response gradient from segments 3 - 9. This can be explained by considering the antenna as a simple core conductor (a cylinder of cuticle containing conducting haemolymph) of constant resistance per unit length. The negative EAG potential is generated in the haemolymph of the terminal three segments (demonstrated above), and this results in a potential gradient between segment 9 and the indifferent electrode in segment 2. A similar potential gradient was found along the antenna of Ostrinia nubilalis by Nagai (1981), but in this moth the olfactory sensilla are distributed evenly on all segments of the antenna. This led Nagai to conclude that the EAG was "a summated result of the responses from the receptors on the antennal region between the recording and indifferent electrodes". In O. surinamensis,

however, EAG responses were recorded from segments 3-8 where there were no receptors between the electrodes. In an elegant series of experiments where he recorded from areas masked from stimulation, Nagai was able to identify a slow electrotonic potential which spread

along the antenna from the stimulated region, but this did not resemble the rapid depolarisations recorded here.

The responses of the terminal 3 segments are less readily explained. The results of Nagai (1981) and the demonstration above that there are CD receptors on all 3 club segments predict that the EAG amplitude should rise significantly from segment 9-11 as more receptors are included between the electrodes, but this is clearly not so. In contrast, two previous studies have reported a similar result to the above. In I. ni, Payne et al (1970) recorded a higher EAG response from the middle than from the base of the intact antenna, yet the response from the tip was not different from the response from the middle. Similarly in Pieris brassicae (Behan and Schoonhoven, 1978), the EAG response was found to increase from segments 12-30, but little, if at all, from 30-36. These terminal segments comprise the clubbed tip of the butterfly, and single cell recordings together with SEM studies showed that numerous sensilla responding to the odour stimulus were actually present.

A possible explanation of the similar responses recorded from the club segments of both O. surinamensis and P. brassicae is that the enlarged haemolymph channel in these segments offers a path of negligible resistance between them as compared with the higher resistance of the antennal shaft. In P. brassicae, Behan and Schoonhoven (1978) suggested that the enlarged club segments "caused shunting". Unfortunately, the resistance of the antennae was not measured in either of the above experiments.

The results from the experiments on O. surinamensis appear to be contradictory, since segment removal suggested that the receptors

of the terminal 3 segments were connected in series, whilst the response gradient predicted by this was not shown in the club segments of the intact antenna. Kaissling (1971) proposed that the EAG was summed from the receptor potentials of many sense cells lying in series. Each cell acted as a dipole, and acted in series due to the orientation of the cells in the epidermis of the Saturniid antenna. However, for a complete explanation of the production of the EAG response, it is necessary to consider the actual position of the electrodes during recordings, since several different methods have been used to obtain an "EAG response". In detailed studies of the generation of individual receptor potentials (Kaissling, 1971; Kaissling and Thorson, 1980) an electrode was placed over a cut olfactory sensillum, i.e. in contact with the receptor lymph. Nagai(1981) also recorded from the antennal surface with moist filter paper strips, which presumably made electrical contact via pores in the olfactory sensilla. In all three experiments that found no increase in response over the terminal antennal segments (above; Payne et al, 1970; Behan and Schoonhoven, 1978), glass electrodes were used to penetrate the antenna, allowing recording from the haemolymph.

To examine the effect of such different methods, a simple theoretical model was devised and then constructed from electrical components (Fig. 17b). The receptor cell potentials were simplified as DC cells, connected in series, and the antenna as a core conductor with an outer conducting layer (epidermis) and an inner conducting core (haemolymph), separated by a high resistance layer. The resistance between the haemolymph of the terminal segments was negligible compared to that between segments 2-9. When constructed, the model produced similar results to the experiments reported above.

Recording from the "haemolymph" of each "segment", using a DC voltmeter, gave a potential gradient from segments 2-9, but no difference from segments 9-11. Progressive removal of segments 11, 10 and 9 caused sequential reductions, eventually to zero, of the potential recorded in the haemolymph of segment 8. Furthermore, the model produced a rising potential gradient in the "epidermal" layer of the club segments, which agrees with the findings of Nagai (1981).

Although the model is highly simplistic, for example in treating neurones as simple DC cells, functionally it does resemble the antenna of O. surinamensis and may be applicable to other insects. Additional measurements, such as of the resistance of each antennal segment, will test the model further.

CHAPTER 4.

ANTENNAL AND BEHAVIOURAL RESPONSES TO FOOD VOLATILES.

INTRODUCTION.

Many phytophagous insects locate host plants using plant odours as olfactory cues (for examples see the review by Visser (1986)). Adult grain beetles are no exception. Both sexes of a number of species that feed as adults have been shown to respond to a variety of host (i.e. food) odours (Freedman et al. ., 1982; Mikolajczak et al., 1983, 1984; Stubbs et al. ., 1985; Pierce et al. ., 1981; Yamamoto et al. ., 1976). Consequently, food odours offer the basis for a detection and monitoring system for stored-product insect pests (Pinniger, 1975; Levinson and Levinson, 1979).

One stored-product that has been shown to be particularly attractive to insects is carob (seed pods from the carob tree Ceratonia siliqua (Leguminosae)). Bait bags containing carob have collected over 40 species of stored-product insects and mites (Pinniger and Wildey, 1979), and subsequent work has attempted to identify the components of carob which are responsible. Both extracts and a vacuum distillate of carob pods were found to be attractive to the saw-toothed grain beetle in laboratory assays, and some active components have been identified (O'Donnell et al. ., 1983; Stubbs et al. ., 1985). However, no investigations into the perception of food odour in grain beetles have been attempted.

A number of possible mechanisms for host recognition using odours

have been suggested, depending on the nature of the plant volatiles produced (Visser, 1986). Some plant odour components are highly specific, so insects can recognise plants by developing receptors for such key components. Crucifers, for example, typically release allylisoithiocyanate, which has been shown to attract several crucifer-feeding insects including the cabbage root fly Delia brassicae (Finch and Skinner, 1974). In turn, the fly has been shown to possess antennal receptors for this component (Wallbank and Wheatley, 1979). Alternatively, odour recognition may involve perception of several "general" plant odour components. Species of Drosophila, for example, are attracted by volatiles produced during fermentation (Hoffmann and Parsons, 1984), whilst "green odour" components (leaf alcohols and aldehydes) cause both behavioural and antennal responses in a range of species, including Leptinotarsa decemlineata (Visser, 1979, 1983; Visser and Ave, 1978; Ma and Visser, 1978) and Psila rosae (Guerin et al., 1983; Guerin and Stadler, 1982; Guerin and Visser, 1980).

Previous work has shown that carob volatiles cause attraction of O. surinamensis in a behavioural assay (Stubbs et al., 1985), and also elicit a consistent EAG response (Chapter 3). The aim of this work, therefore, was to identify the component(s) responsible for both the EAG and behavioural responses, and so determine whether single or multiple components are involved, and whether these are of a specific or general nature. A new behavioural assay was also developed to measure "attractancy" (Shorey, 1977) to olfactory stimuli.

Not only O. surinamensis responds to carob odour, however. The broad range of species that have been caught in carob-filled bait bags

poses the further question of whether these additional species respond to the same, or to different components of the carob odour. Consequently, to answer this, the volatile components involved in perception of carob odour in a second species, the foreign grain beetle Ahasverus advena (Coleoptera: Silvanidae), were also determined.

MATERIALS AND METHODS.

Insect rearing.

Saw-toothed grain beetles for use in both EAG and behavioural tests were cultured on a 5:5:1 mixture of rolled oats, whole wheat flour and brewer's yeast in 1.0 litre glass jars at 25^oC in a 16h light: 8h dark lighting regime (lights on 1800hrs, lights off 1000hrs). Insects were used 4 weeks after emergence as adults. Individuals were removed from cultures 24h prior to use, and deprived of food and water overnight in empty 5cm x 2.5cm glass tubes (20 insects per tube). Foreign grain beetles (Ahasverus advena) were cultured and treated in the same way.

Materials tested.

Crushed carob pods were obtained from Cypriot carob trees (Ceratonia siligua : Leguminosae) and heated to 70^oC for 7h, twice, to kill any insects already present.

Carob distillate (CD) was prepared by heating this carob, finely ground, for 3h under reduced pressure and collecting the distillate (for further details see Stubbs et al ., 1985). Such treatment produced approximately 1.4ml of colourless, sweet-smelling, aqueous distillate per gram of carob.

Carob distillate was further separated into acidic, neutral and basic fractions in ether, using the extraction scheme of Stubbs et al.

(1985), which gave each fraction at the concentration found in the original carob distillate. Analysis of such fractions by Gas Chromatography- Mass Spectroscopy (GC-MS) (electron impact) in the same earlier study identified the major components as follows:- Acidic fraction: acetic acid (375 ng/ul), isobutyric acid (2340 ng/ul),

n-butyric acid (200 ng/ul), 2-methyl butyric acid (155 ng/ul) and n-hexanoic acid (240 ng/ul); Neutral fraction: 2-furaldehyde (18 ng/ul) plus 4 unidentified components; Basic fraction: 2-furaldehyde (7 ng/ul) plus 2 unidentified components.

AnalaR grade samples of the above 6 identified components were obtained for testing.

Collection of carob volatiles by aeration.

Carob volatiles were also collected by a different technique - aeration - which did not involve heating, and resulted in a non-aqueous extract. The apparatus used was similar to that described by Pierce et al . (1984b) (Fig. 18). Filtered and humidified air was drawn through an aeration vessel containing c.1kg of crushed carobs at 2 litres/min., and the volatiles removed from the effluent using a trap filled with Porapak Q. The aeration was continued for a total of 434 hours (giving a total of 400,000 gram hours (gh)), at the end of which the Porapak was extracted with 250ml of pentane in a Soxhlet apparatus for 48h and the extract concentrated down on a rotary evaporator to give 1ml of extract A7E1.

GLC analysis of aeration extracts.

Aeration extract A7E1 was analysed using a Varian 3700 Gas-Liquid Chromatograph (GLC) fitted with a flame ionisation detector (FID) and a 50m x 0.32mm i.d. fused silica capillary column coated with chemically bonded OV-1 (Phase Separations Ltd.). Helium was used as the carrier gas at an injector pressure of 1.5 kg/sq.cm. Injections were made in the splitless mode, with the oven initially at 40°C for 1min., then programmed to rise at 10°C/min to 220°C (injector temp.

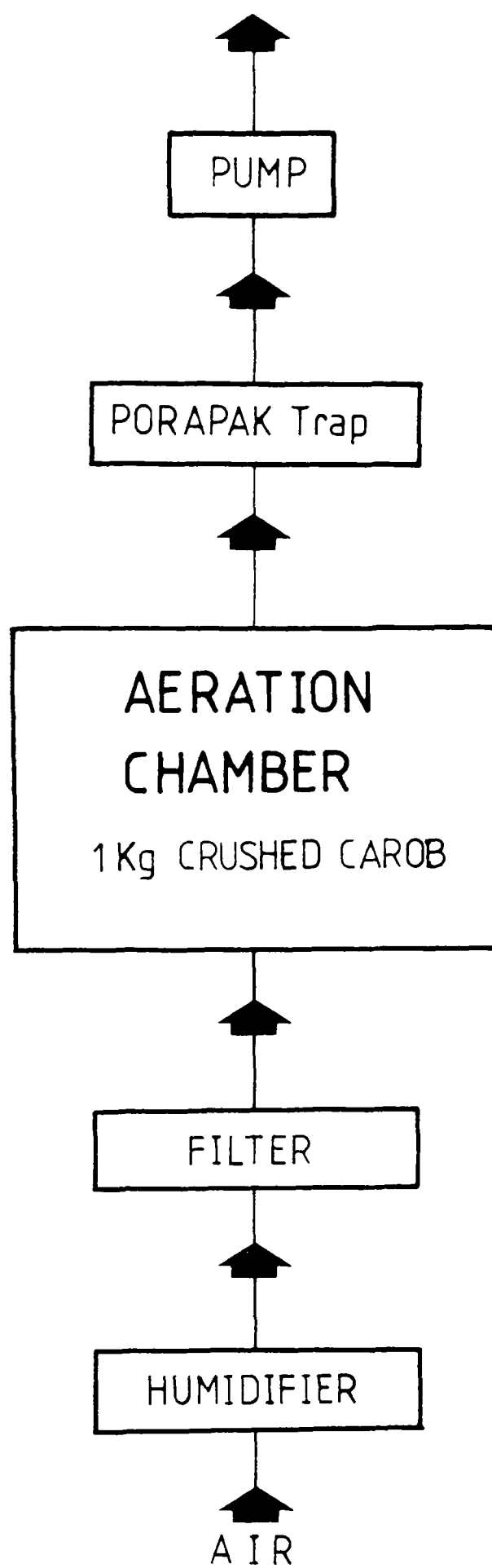


FIG.18. Aeration apparatus for collection of carob volatiles.

200°C, detector temp. 240°C).

Electroantennogram (EAG) recordings.

The standard EAG procedure described above (Chapter 3) was used to determine the amplitude of the response to the various chemicals, mixtures and extracts. Individual preparations were set up and left for 30min. to settle before presentation of the odour cartridges. Standard stimuli of 5ul of carob distillate were interspersed between every 2-3 tests, and each test was preceded by a blank presentation of the relevant solvent. The EAG response to any stimulus was determined as the maximum deflection of the DC trace from the baseline during the stimulus, from which was subtracted the blank response to control for the mechanical disturbance of the solenoid on the airflow, and any effect of the solvent. The resulting value was expressed as a % of the average of the 2 standard presentations (CD) bracketing the test. This, theoretically, controlled for differences in sensitivity both between preparations, and within the lifespan of a single preparation, but in addition, where direct comparisons were required, e.g. between different fractions, all were presented to the same individuals.

Preparations of O. surinamensis were presented with cartridges containing crushed carob, carob distillate and its acidic, neutral and basic fractions, identified components of CD and the aeration extract A7E1, with a minimum of 5 replicates of each sex tested with each material. Preliminary EAG recordings were also made with Ahasverus advena to determine the response to the aeration extracts.

Coupled Gas Chromatography-Electroantennography (GC-EAG).

The carob volatiles responsible for the EAG responses to the

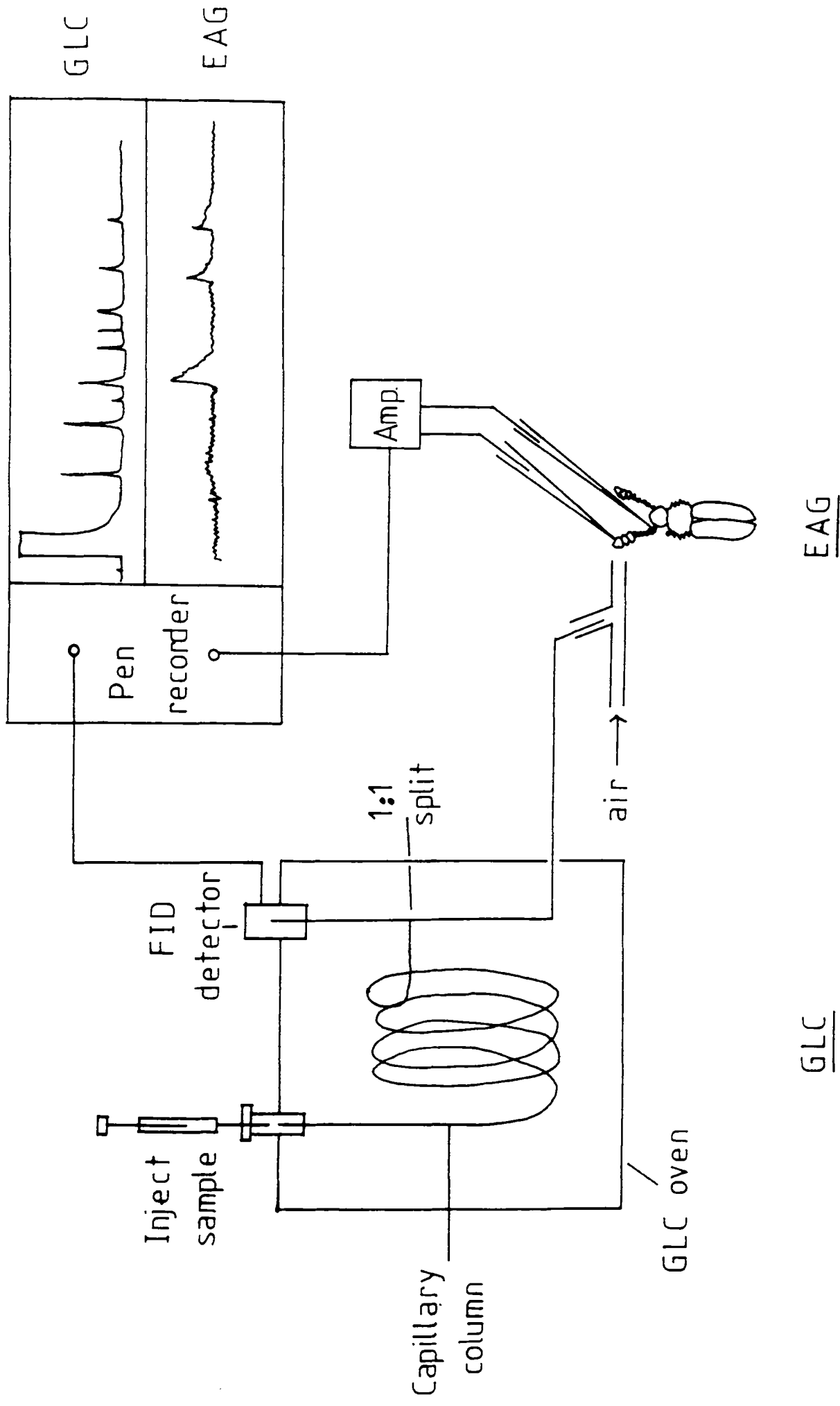


FIG.19. Coupled GC - EAG apparatus.

aeration extracts in both O. surinamensis and A. advena were determined by coupled GC-EAG. This technique, introduced by Moorhouse et al . (1969), allows the components of a complex mixture to be separated by GLC, and then presented separately to an insect's antenna as they elute from the column. (For a recent review of the technique see Struble and Arn, 1984).

The system developed here resembled that described for GC-single cell recordings by Wadhams (1984) (Fig. 19). Within the GLC oven the column effluent was split 1:1 between two 50cm lengths of silica capillary, using a glass-lined T-piece. One capillary was connected to the FID, whilst the other ran from the oven, via a heated jacket (at 220°C), to the EAG odour delivery line.

Outputs from the GLC detector and the EAG were recorded simultaneously using a 2-channel chart recorder. Several runs were made with both sexes of each species, and the peaks that consistently produced EAG responses determined. Initial attempts were made to chemically identify these components using a Finnigan 1020 GC-Mass spectrometer (GC-MS).

Behavioural assays.

A behavioural assay was developed to investigate the response of insects to olfactory stimuli in still air (as opposed to moving air e.g. in a wind tunnel) to approximate to the likely conditions found within bulks of stored grain.

Previous work on such responses of stored-product insects have utilised almost as many bioassay designs as there have been studies (e.g. Pinniger and Collins, 1976; Levinson and Bar Ilan, 1970; Phillips and Burkholder, 1981). The assay used here was modified from

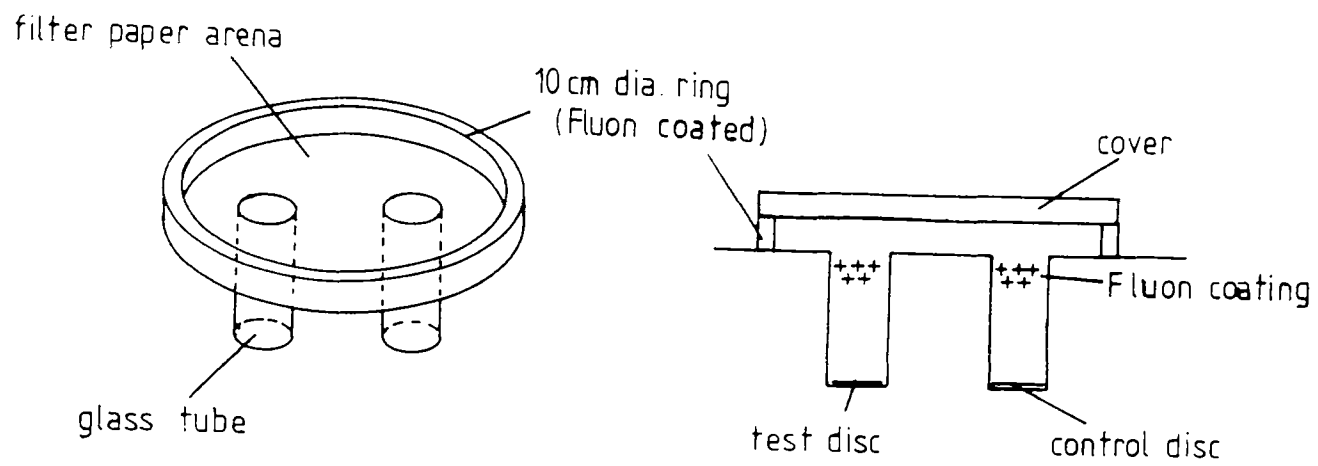


FIG.20 (a) Two-choice pitfall bioassay apparatus.

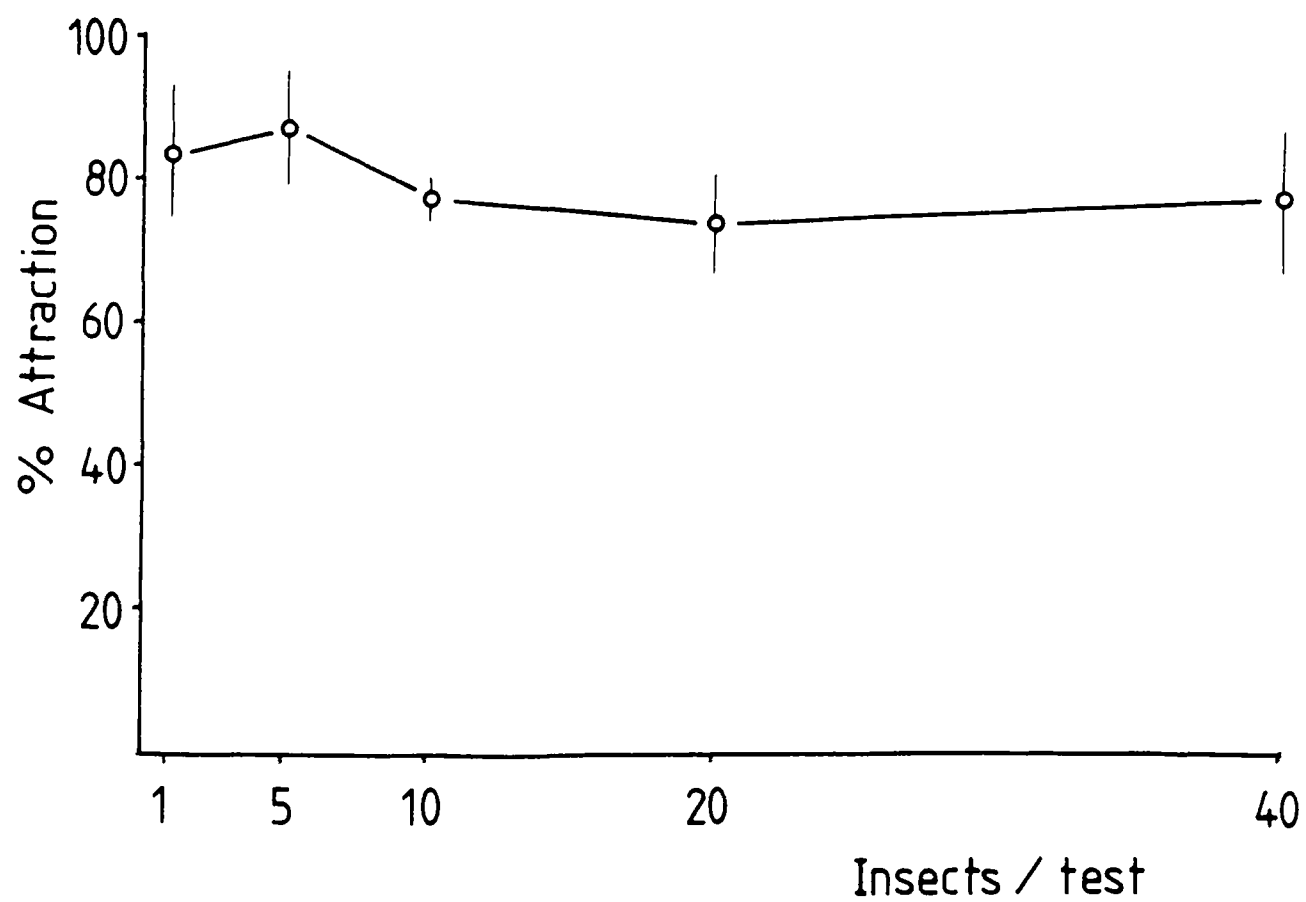


FIG.20 (b) Effect of the number of insects used per test on the attraction to Carob Distillate in the 2-choice pitfall bioassay. (80 insects used in each case, means $\pm$ SE).

the 2-choice pitfall test of Pierce et al . (1981). Two 5cm x 2.5cm glass tubes were glued below holes cut in an 11cm diameter filter paper disc, which was supported on a metal base plate (Fig. 20a). In contrast to the assay of Pierce et al . (1981), however, the tube necks were coated in Fluon, so that once an insect entered a tube it could not leave. This apparently minor modification had a significant effect on the results (see below). A 10cm diameter perspex ring, also coated with Fluon, was placed on the filter paper and the arena covered with a glass plate.

The test insects (20 per test) were introduced into the arena and allowed 10 mins to settle. The test and control stimuli were then applied to 1.5cm diameter filter paper discs (in a fume hood in another room) and after allowing 30s for the solvent to evaporate, the discs were introduced into the relevant pitfall tubes. The arenas were then covered and the tests run for 1 hour at 25^oC. All tests were set up under red light and run in darkness between 1400 and 1800hrs (i.e. during the scotophase), since saw-toothed grain beetles kept under a similar lighting regime to that used here have been reported to show a peak of activity during this period (Bell and Kerslake, 1986).

In the absence of olfactory stimuli insects typically remained either stationary or walked slowly around the arena's perimeter. On introduction of an odour source, insects generally responded by waving their antennae, and moving in a series of zig-zags towards the test pitfall. On encountering the Fluon collar insects halted, and only if they continued towards the odour source did they fall into the pitfall. Thus only insects making a positive oriented response to the source across the Fluon were counted in this assay, giving a measure

of "attractancy" and not "arrestance" (Shorey, 1977).

At the end of the test period, the numbers of insects in each pitfall tube and those not responding were counted. For each arena, the % attraction to the test material was calculated as $100(T-C)/N$ where T= no. of insects in test pitfall tube, C=no. of insects in control tube and N= total no. of insects tested (Tamaki *et al.*, 1971; Nara *et al.*, 1981). A minimum of 5 replicates per concentration of each material were tested, in a totally randomised design.

Analysis of results.

Differences between responses to test and control stimuli were tested for significance using a Chi square test, computed as $\chi^2 = (T-C)^2 / (T+C)$ (Mikolajczak *et al.*, 1984). The attraction to different materials was compared using the mean % responses. This value could vary from +100% (all insects in test pitfall tube) through 0% (equal numbers in test and control tubes) to -100% (all insects in control tube), and as the distribution of values followed a near normal distribution, parametric tests (2 way or factorial analyses of variance (Sokal and Rohlf, 1969)) were used to determine differences between the responses to different materials, or between the responses of different groups of insects.

Validation of behavioural bioassay technique.

Before use, the 2-choice pitfall assay described above was critically examined to determine the effect of (i) applying Fluon to the tube necks (ii) varying the number of insects used in each test and (iii) whether the results are affected by pheromone mediated

aggregation.

(i) The effect of applying Fluon to the pitfall walls on the observed response.

The response of insects (20 per test) to 5ul of carob distillate versus 5ul of water (control) was measured using pitfalls either with or without Fluon, using 10 replicates of each. In the Fluon coated tubes, (insects could not escape from tubes after entering) the attraction measured to carob distillate was $60.0 \pm 3.8 \%$, whilst in uncoated tubes it was $5.0 \pm 6.3 \%$. The Fluon was therefore essential for demonstration of attraction to carob distillate, since without it insects could climb out of the pitfalls.

(ii) The effect of test insect numbers on the observed response.

The majority of bioassay techniques utilise multiple-insect testing, and whilst this allows for the rapid accumulation of data, each insect's behaviour may not be independent of the other insects in the arena. Pierce *et al.* (1983), for example, reported that attraction to odour sources varied with the number of insects used per test, so this effect was investigated.

The % response to 5ul of carob distillate was measured at 5 different test insect densities (1, 5, 10, 20, and 40 per arena) testing a total of 80 insects in each case. The results (Fig. 20b), however, showed little effect of insect density on their response, suggesting that insects responded independently to the odour source, even in multiple-insect tests. A density of 20 insects per test was selected for further work, since this provided a sufficiently accurate estimate of attraction in each arena, yet allowed for several

replicates to be run with the beetles available.

(iii) Possible effects of pheromone-mediated aggregation.

O. surinamensis produces a pheromone that causes both sexes to aggregate (see Chapter 5). This could markedly affect any multiple-insect assay as once insects are attracted to an odour source, they could then attract other individuals by subsequent pheromone production. To investigate whether this occurred in the assay used here, 10 insects (mixed-sex) were placed in one pitfall and the test run as before with 20 responding insects in the arena. After one hour, the numbers of insects in each tube were recounted to determine whether more insects entered the insect-occupied, as opposed to the empty tube. The % attraction to the tube containing insects was $3.8 \pm 6.3\%$ ($n=6$), with no significant difference between test and control responses ($\chi^2=0.32$)

In some grain beetles pheromone production is dependent on the presence of food (Pierce et al. ., 1984b), so the above experiment was repeated with 10 beetles on 5g of fresh culture medium versus culture medium alone in the pitfall tubes. Again there was no significant difference between the numbers of insects entering either tube ($\chi^2=1.05$, % attraction = $-8.0 \pm 12.3\%$).

Insects which fall into a pitfall during a test do not, therefore, appear to affect the behaviour of the other insects in the arena.

RESULTS.

EAG responses to fractions and components of Carob Distillate.

Male and female *O. surinamensis* showed similar responses to 0.5g of ground carob in a cartridge (males=497 $\pm$ 70 uV, n=22; females=460 $\pm$ 59 uV, n=18). The EAG response to carob distillate is shown in Figure 21a. The response of males to 5ul of carob distillate (used as the standard) was 663 $\pm$ 66 uV (n=60), and that of females 569 $\pm$ 32 uV (n=61).

Analysis of the results (by ANOVA) showed significant differences between the EAG responses to the acidic, neutral and basic fractions ($F=189.3$, $P<0.001$) (Fig. 21b) but as with carob distillate, there were no differences between the responses of males and females. Of the individual fractions, the neutral produced the largest EAG response at each concentration tested, whilst the basic generally gave the lowest response. Presenting the 3 fractions together (A+N+B) increased the magnitude of the response, but even the mixture did not give an EAG response comparable with that to the original carob distillate. At the same concentration, the fractions together produced only about 60% of the full CD response, suggesting that some components had been lost in the extraction process.

The EAG responses to the individual identified components were also low compared to the standard, even at the highest concentrations tested (Fig. 22). The dose response curves were essentially similar for each chemical, although the antennae appeared to be most sensitive to hexanoic acid, since this had around a 10-fold lower threshold. Some differences were also found at higher concentrations than those shown in Figure 22. Presentation of 5000ug of acetic, butyric or

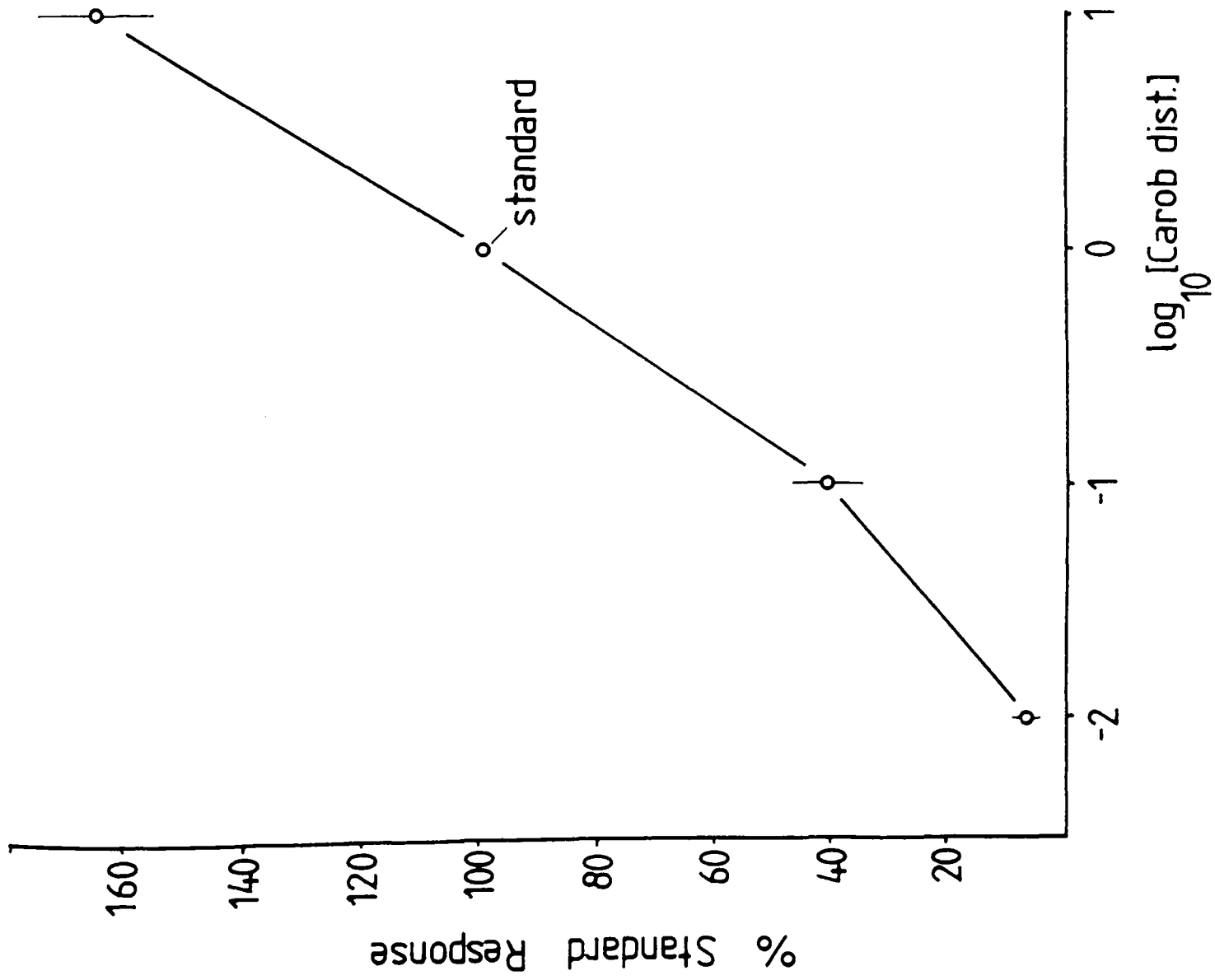


FIG.21 (a) EAG response to Carob Distillate.
(Mean $\pm$ SE, $n=10$)

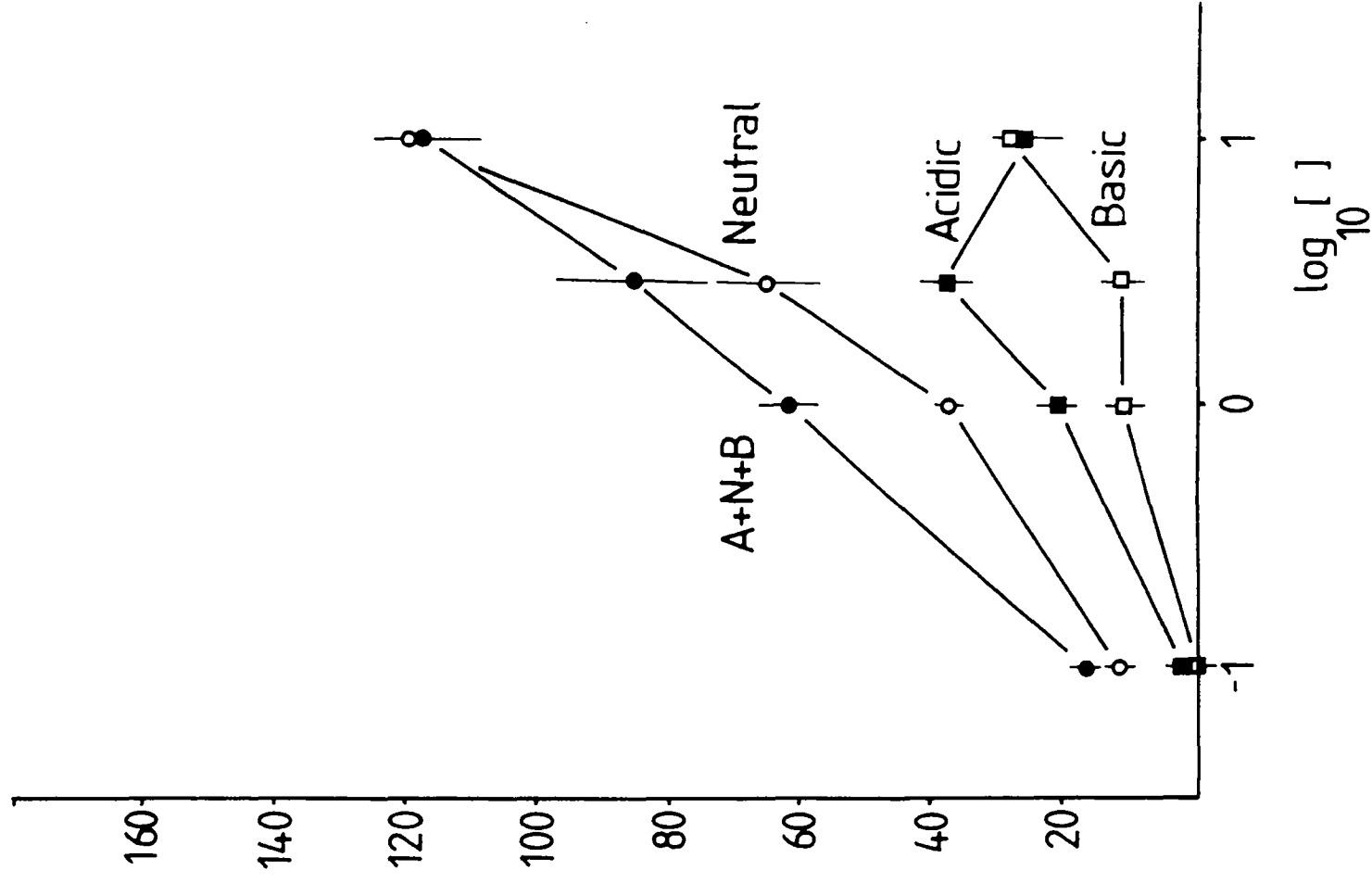


FIG.21 (b) EAG responses to fractions of Carob Distillate. (Concentrations relative to CD; mean $\pm$ SE, $n=10$).

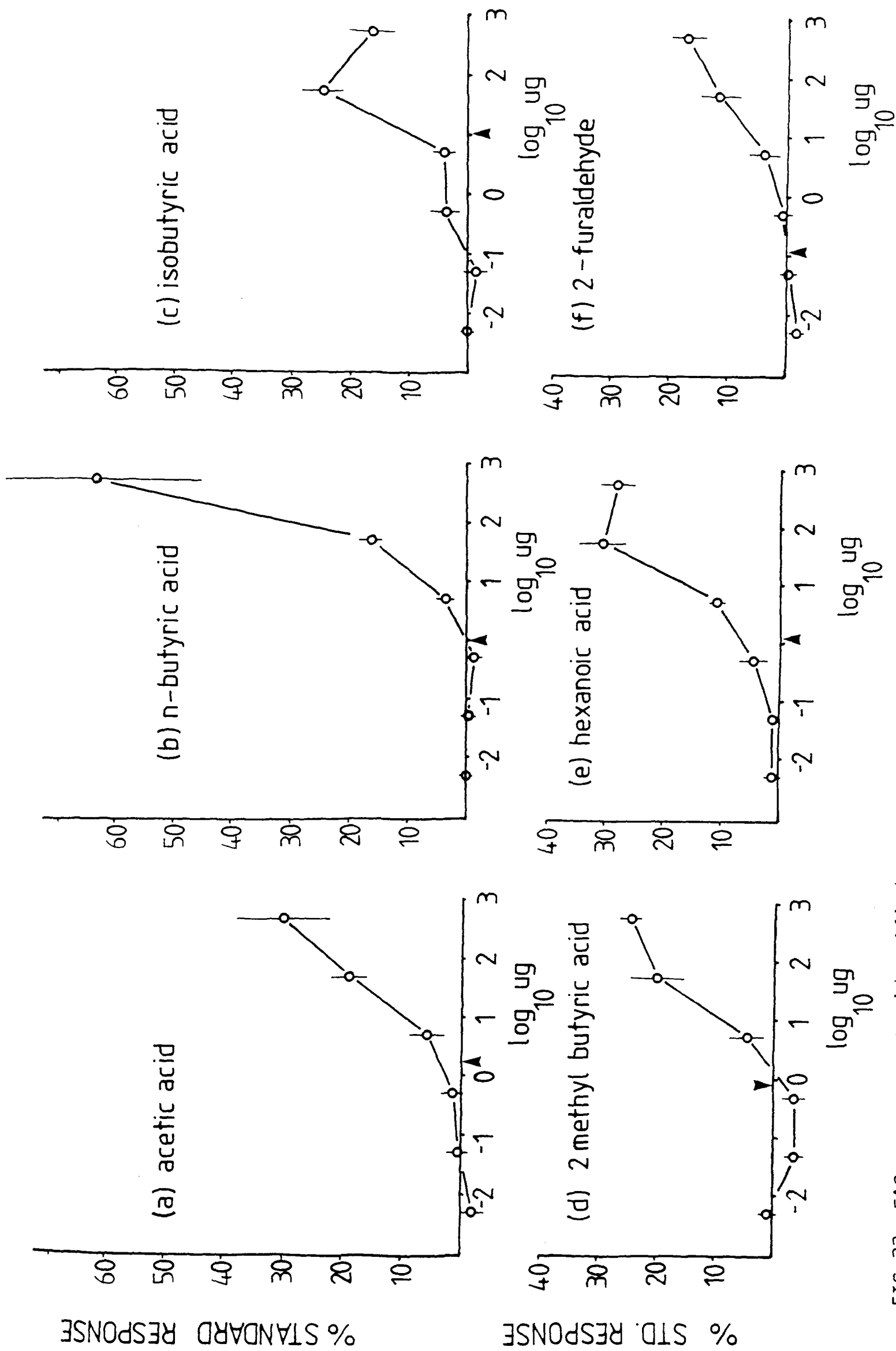


FIG-22 EAG responses to identified components of Carob Distillate. Responses calculated as % of standard response (5ul of Carob Distillate). Arrow in each figure shows the amount of each component in 5ul of Carob Distillate. (Means $\pm$ SE, $n=10$, 5 males, 5 females.)

isobutyric acids often produced large magnitude hyperpolarisations of the antennal tip (as opposed to the normal depolarisation). However, such responses were not repeatable with the same preparation, so probably caused permanent damage to the olfactory receptors.

The arrows in Figure 22 show the amount of each component reported to be present in the CD standard (Stubbs et al., 1985). When all 6 compounds were combined in these amounts in either water or pentane they produced EAG responses of $15.1 \pm 1.8 \%$ ($n=14$) and $22.1 \pm 2.1 \%$ ($n=32$) of the standard EAG response respectively. Therefore, again, the component(s) responsible for the majority of the EAG response to carob distillate was missing. An attempt to identify the EAG active components in the neutral fraction by GC-EAG was unsuccessful, so further attempts at such identifications were concentrated on the aeration extract.

EAG responses to carob aeration extract.

O. surinamensis.

Presentation of 400gh of aeration extract A7E1 gave a mean EAG response of $606 \pm 82 \text{ uV}$ ($n=12$). GLC analysis of the extract (Fig. 23) demonstrated the presence of a large number of volatile components, and GC-EAG traces showed that at least 3 peaks consistently produced EAG responses in both sexes that could be distinguished from background noise and baseline drift (Fig. 23). Of these, the large peak P was identified by GC-MS as n-hexanoic acid, whilst the remaining peaks have yet to be identified and subsequently tested for EAG activity.

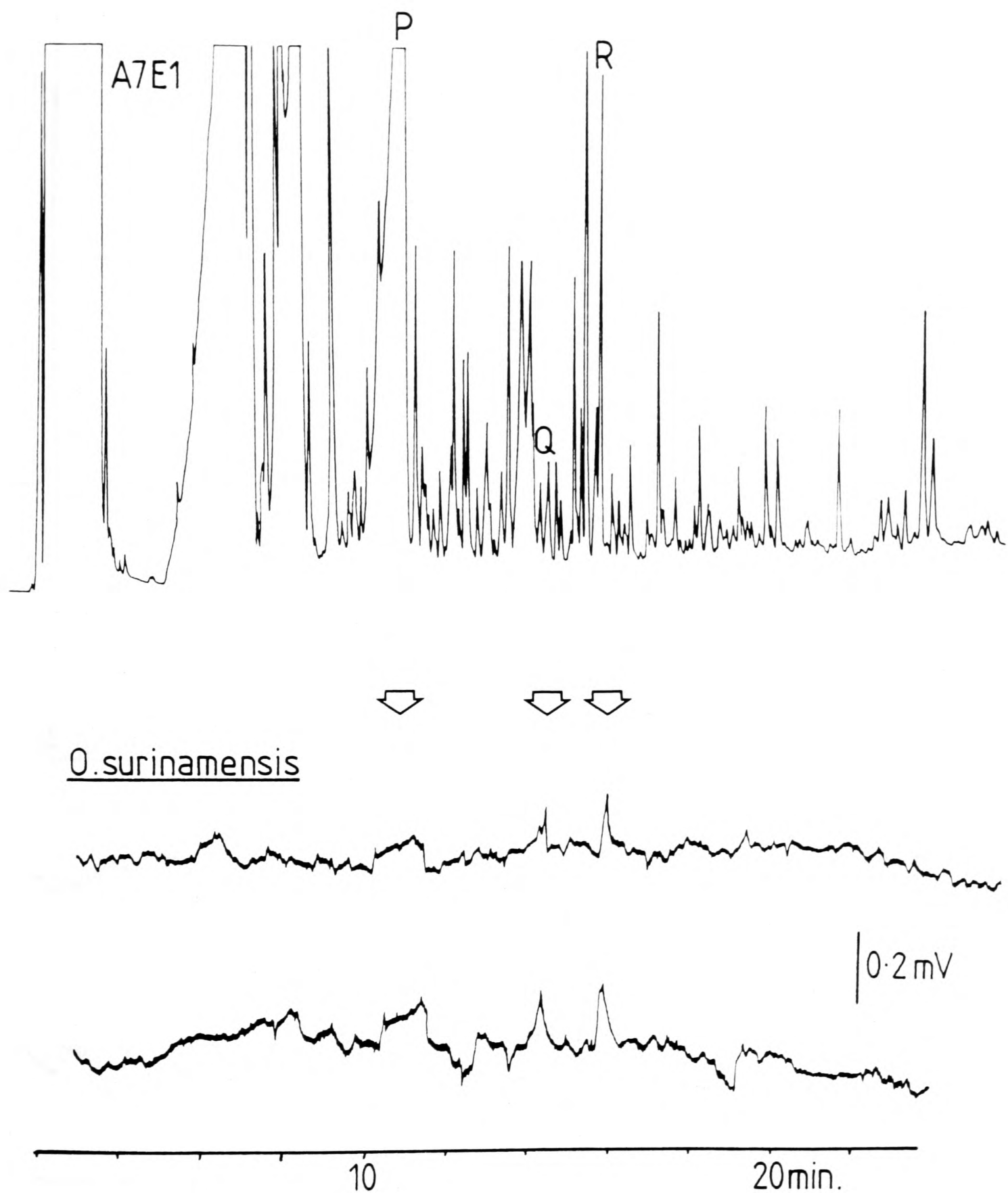


FIG.23 Coupled GC-EAG trace for O. surinamensis response to Carob volatiles A7E1, showing 3 EAG responses (open arrows).

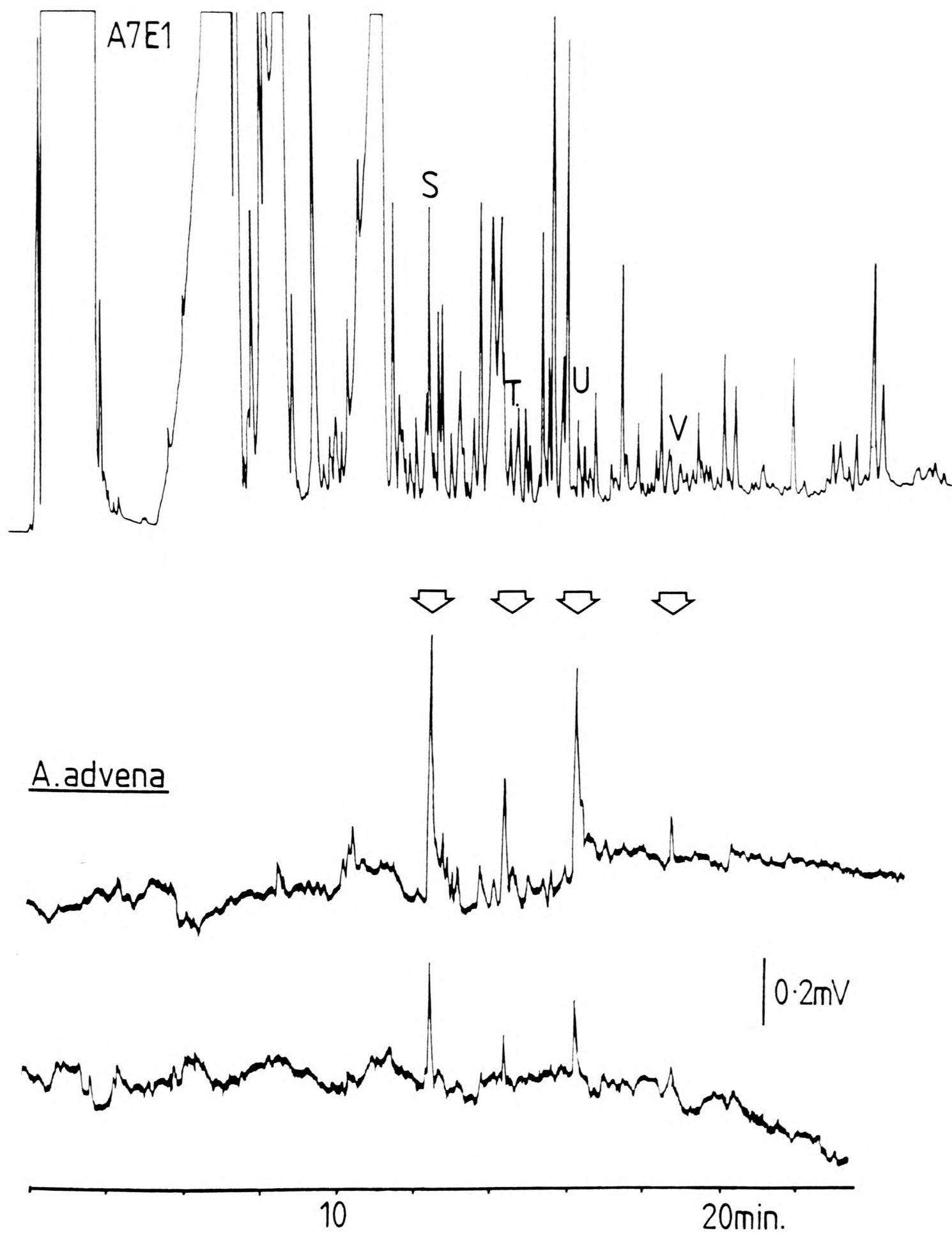


FIG.24 Coupled GC-EAG trace for Ahasverus advena response to Carob volatiles A7E1, showing at least 4 EAG responses (open arrows).

Ahasverus advena.

Presentation of 400gh of extract A7E1 produced a larger amplitude EAG response in this species (1330 ± 204 uV, $n=5$). GC-EAG traces for this extract showed very clear and consistent EAG responses to at least 4 components (Fig. 24) One of these (T in Fig. 24) had an identical retention time to the component Q which caused a response in O. surinamensis. Chemical identification of these components have yet to be completed.

Behavioural response to carob distillate and fractions.

Pitfall assays showed that O. surinamensis was significantly attracted to carob distillate, down to 1/10 of the normal CD concentration (Fig. 25a). Each of the fractions also produced significant attraction, at least at higher concentrations (Fig. 25b), but there were significant differences between their effects ($F=6.9$, $P<0.01$). Overall, the neutral was more attractive than the acid or basic fractions ($P<0.05$, Fisher's protected LSD test, Sokal and Rohlf, 1969), whilst the mixture was slightly, but not significantly, more attractive still. At the 2 highest concentrations tested, the mixture of fractions was as attractive as the original CD, but less so when diluted.

Behavioural response to carob aeration extract.

Both species were found to be strongly attracted to a 400gh dose of the extract A7E1. The level of attraction of O. surinamensis was 80.0 ± 4.8 % ($n=6$), whilst that for A. advena was found to be 76.7 ± 7.1 % ($n=6$).

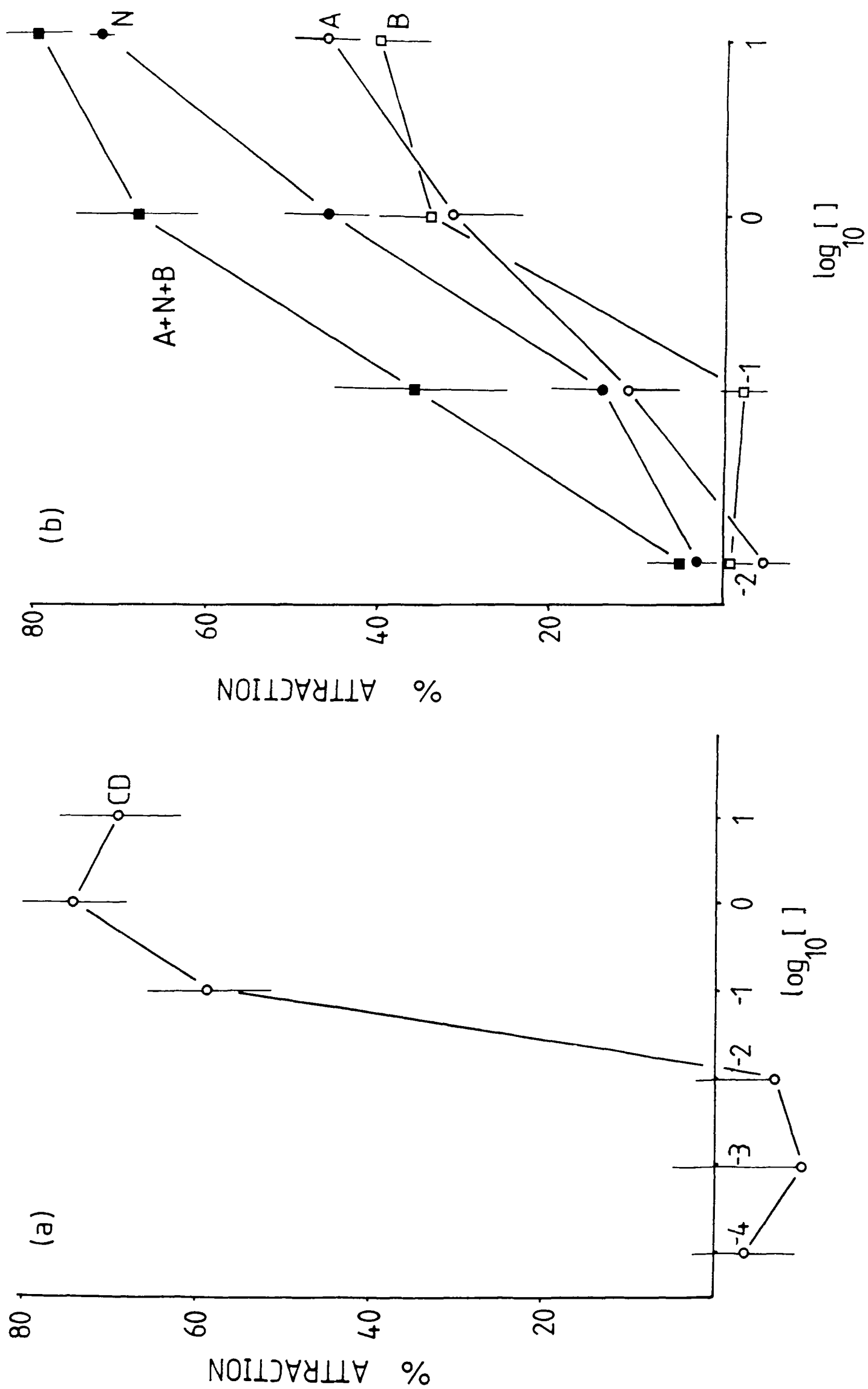


FIG.25 Behavioural response of insects to (a) Carob Distillate and (b) fractions of CD (A-Acidic, B-Basic, N-Neutral) in 2-choice pitfall bioassay. 5ul of each concentration used in each case, Concentrations given relative to Carob Distillate. (Means $\pm$ SE, $n=6$).

DISCUSSION.

Behavioural bioassays.

Previous assays of odour responses in stored-product insects have utilised several designs. The earlier work on the response of O. surinamensis to carob volatiles and water used an "insect activity detector", in which the number of crossings made by insects under a suspended wick emitting the test material was counted by a light dependent resistor (Pinniger and Collins, 1976; Stubbs and Griffin, 1983; O'Donnell et al. ., 1983; Stubbs et al. ., 1985). The number of crossings gave a measure of the "attractancy" of each material. Other assays have used open arenas, again with a central odour source (Levinson and Bar Ilan, 1970). Insects were released into the arena, and the numbers of insects present at the source counted either during, or at the end of the test. Pierce et al. . (1981) devised a 2-choice pitfall assay, on which the present method was based.

Each of the above methods, however, fail to distinguish whether a test material acts as an "attractant" or an "arrestant". Shorey (1977) defined an attractant as "a chemical that causes animals to make oriented movements towards its source", whilst an arrestant "causes animals to aggregate near the chemical source by decreasing the speed of locomotion or appropriately affecting the rate of turning". These concepts of olfactory "attraction" and "arrestment" have been criticised by Kennedy (1978) as teleological, and because they do not define the actual mechanisms involved (see Kennedy, 1986). However, a full analysis of the orientation mechanisms was not considered essential for the present study.

In the above bioassays, large numbers of insects at the odour source at the end of a test could be achieved by an attractant, an arrestant or a combination of the two. This applies equally to the 2-choice pitfall assay used by Pierce *et al.*, (1981) since insects such as *O. surinamensis* are able to walk on vertical glass surfaces, and so can leave a pitfall after entering. Thus, if an odour acts initially as an attractant, but not as an arrestant, insects may leave the test pitfall and no effect is recorded at the end of the test. This could explain the importance of the Fluon in the design described here.

A second possible criticism of some assays (e.g. Pierce *et al.*, 1981) is that the insects are allowed to contact the odour source during the test, and consequently such tests cannot distinguish between the roles of olfactory and gustatory cues in producing a response.

In the assay technique reported above, insects could not contact the odour source before entering a tube, so only olfactory responses were measured. The test should also have measured attractancy and not arrestance. It could be argued that a klinokinetic response near the rim of the pitfall tube may have caused beetles to fall into the trap without having shown truly oriented (tactic) movement. Observation of beetles suggested that this was not the case, however, and that directed movement, thus attractance, was the main mechanism responsible. A further advantage of this design, on the applied side, was that it more closely resembled the trapping situation in the field (e.g. in a probe trap (Burkholder, 1984a,b)) where insects cannot leave a trap once they have entered.

Antennal and behavioural responses.

Strong correlations were shown between the relative EAG and behavioural responses to the various materials tested. Those eliciting large EAG responses (carob distillate, aeration extract A7E1) subsequently proved to be highly attractive, in both species tested, and similarly, within the CD fractions tested, there was close agreement between the relative EAG response and the level of attraction produced.

The neutral fraction proved to be the most active of the three in both EAG and behavioural tests. In the insect activity detector assay, Stubbs et al . (1985) also found the neutral fraction to be active at high concentrations, but less so than the acidic fraction. The reason for the relatively greater response of the neutral fraction in the present assay is not clear, but may reflect the different assay methods used. The previous study also reported that, when presented at concentrations above that found in carob distillate, all 3 fractions were initially repellent, but no repellency was observed in the present assay, even at the maximum concentrations.

It was not possible to identify the component(s) in the neutral fraction responsible for the EAG response, however. The only identified material, 2-furaldehyde, elicited little EAG response at the relevant concentration, and has been found to have little effect on behaviour (Stubbs et al ., 1985).

Of the single components tested, hexanoic and isobutyric acids produced the largest EAG responses at the concentrations found in

carob distillate, and hexanoic acid was also identified as an EAG-active component of carob odour by GC-EAG. Previous behavioural work identified hexanoic acid as the most attractive single component, but isobutyric acid was found to have a repellent effect (Stubbs et al., 1985). Hexanoic acid has also been reported to elicit behavioural responses in several other species of stored-product insects. Yamamoto et al. (1976) identified hexanoic acid as the major attractant in rice and corn for Sitophilus zeamais. Levinson and Levinson (1978) suggested, however, that such short chain fatty acids were generally repellent to stored-product insects, and such an effect has been reported in Trogoderma granarium, Tribolium castaneum and larval Dermestes maculatus (Cohen et al., 1974).

The hyperpolarisation of the antennal tip produced by high concentrations of acetic, butyric and isobutyric acids were also of interest. Similar responses to short chain fatty acids have been reported in Thanatophilus rugosus (propionic acid) (Boeckh, 1967b) and recently in the stable fly, Stomoxys calcitrans (acetic acid) (Warnes and Finlayson, 1986). In single-cell recordings from the beetle Necrophorus and flies, Boeckh (1962, 1967, 1969) found that fatty acids from C2 - C4 depressed the cell firing rate, whilst acids from C5 - C8 increased it. The present work agreed with this range, since neither 2-methyl butyric acid (C5) or hexanoic acid (C6) caused hyperpolarisation of the EAG. Such responses have been interpreted as inhibitory, and correlated with repellency in behavioural assays, since high concentrations of such acids have been shown to be repellent to Aedes aegypti (Muller, 1968) and acetic acid has been suggested as an inhibitor in the stable fly (Warnes and Finlayson,

1985). However, in the present study, hyperpolarisations were often followed by total insensitivity to further olfactory stimuli, and could have been caused by permanent damage to the receptor membranes, so such interpretations should be made with caution.

The EAG response to the mixture of identified components was well below that to the whole distillate, suggesting that one or more important components were missing. GC-EAG analysis of carob volatiles in the aeration extract, suggested that at least 2 components, apart from hexanoic acid, caused EAG responses. Both have yet to be identified, but the retention times suggest materials of lower volatility than the free fatty acids tested so far.

Comparisons with Ahasverus advena showed that although this species was also strongly attracted to carob volatiles, the antennal response was mainly caused by different components from those active in O. surinamensis. One component elicited responses in both species, but the peaks producing the 2 largest EAG responses in A. advena did not appear to affect the receptors of O. surinamensis.

Further GC-EAG work is in progress to determine whether other species that are attracted to carobs also detect the same range of components, but the above results suggest that for use as a broad spectrum attractant, a complex mixture, rather than a single component, will be required.

CHAPTER 5.

AGGREGATION PHEROMONE: COLLECTION, IDENTIFICATION AND BIOASSAY.

INTRODUCTION

Since the first identification from Bombyx mori (Butenandt et al. ., 1959; Butenandt and Hecker, 1961) there has been a near exponential increase in the number of known insect pheromones. A recent list showed that within the Lepidoptera alone, sex pheromones have been identified for over 300 species, as well as numerous sex attractants (Arn et al. ., 1986). In contrast, however, despite their numerical superiority in species, only about 75 pheromones have been identified to date within the order Coleoptera (Tumlinson, 1985). Most of these have been from bark beetles (family Scolytidae), but several pheromones from stored-product beetles have been identified, and in some cases synthetic pheromones are already available commercially for use in monitoring traps (Burkholder and Ma, 1985).

Observation of the behaviour of O. surinamensis suggests the existence of a pheromone that promotes aggregation, since beetles often clump together in culture jars, either on the surface of the food or on the glass walls (pers. observation). A chemical basis for this is suggested, since if beetle aggregations are removed from the glass walls, they often reform in exactly the same spot.

Pierce et al. . (1981) showed that both sexes of O. surinamensis were attracted to volatiles collected from adult beetles and also from frass, and more recently they have identified 3 compounds produced by male beetles, which together attract both sexes (Pierce et al. .,

1984a, 1984b, 1985). This was achieved by successive fractionation of the volatiles collected, and subsequent behavioural testing.

The following study aimed to collect and identify beetle-produced volatiles, and determine which of these were biologically active, using a different approach - coupled GC-EAG in conjunction with behavioural assays. Such a method has several advantages over the previous procedure. It is possible to determine the antennal response of an individual to every compound in a complex mixture, which would involve enormous effort using other methods. Also, using the technique of progressive fractionation and behavioural bioassay, the presence of a repellent material in a fraction may mask the effect of an attractant.

Beetle and food volatiles were therefore collected and tested in a behavioural assay to determine if they attracted conspecific adults. Beetle-produced volatiles were identified by comparing the odour of beetles on food with the volatiles collected from food alone, and EAG-active volatiles in the mixtures were identified by coupled GC-EAG and subsequent mass-spectroscopy (see Chapter 4). Further behavioural assays were then used to see if the response to the collected volatiles could be explained by the levels of the identified materials present.

The results so obtained should show whether the Slough strain used here produce and respond to the same pheromone components as the Canadian strain previously investigated, and if so, whether the blend ratios are also identical.

MATERIALS AND METHODS.

Collection and analysis of volatiles.

Beetle and food-produced volatiles were collected at M.A.F.F. Slough Laboratory, in a similar manner to that described in Chapter 4. Filtered air was passed through a culture jar containing c. 10,000 adult *O. surinamensis* of mixed sex, feeding on 150g of rolled oats, and the volatiles extracted from the effluent using a Porapak Q trap. Every 2 weeks the culture medium was renewed to prevent the development of larvae in the aeration vessel and the possible subsequent production of larval volatiles. At the same time, the Porapak was extracted using purified n-pentane which was then concentrated down to give 1ml of each aeration extract. Repeated extraction of the Porapak every 2 weeks produced several aeration extracts from each of 3 insect cultures. (These are referred to as A1E1 (aeration 1, extract 1), A4E2 (aeration 4, extract 2) etc.)

Two parallel aerations (A3, A6) were conducted using 150g of rolled oats alone, to determine which of the volatiles were food-produced. All aeration extracts were analysed by GLC using the columns and conditions described above (Chapter 4).

EAG responses to aeration extracts.

To determine whether adult beetles produced any EAG-active volatiles, the EAG responses to combined beetle and food volatiles (extract A4E2) and food volatiles alone (extract A3) were compared. A standard stimulus of 5ul of each extract (equivalent to 1.68 aeration hours) was presented to 20 individuals (10 males, 10 females) in random order. Recordings were made in the standard manner described in

Chapter 3. Results were analysed using a 2 level nested analysis of variance (Sokal and Rohlf, 1969) to identify differences between the responses of individuals to the two extracts, and between the responses of males and females.

Identification of EAG-active volatiles in aeration extracts.

For each of the extracts A1E1, A2E4 and A4E2 (beetles on oats), the components causing EAG responses were identified using the coupled GC-EAG technique (Chapter 4). An attempt was also made to identify the EAG-active components in the aeration extract of oats alone (A3). Each extract was presented to a minimum of 6 individuals, using both sexes, and any GLC peaks producing consistent responses in different EAG traces were subsequently identified by coupled GC-MS.

Purity of synthetic materials.

Three beetle-produced materials were found to give significant EAG responses:-

(Z,Z)-3,6-dodecadien-11-olide (II)

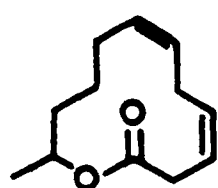
(Z,Z)-3,6-dodecadienolide (III)

(Z,Z)-5,8-tetradecadien-13-olide (IV)

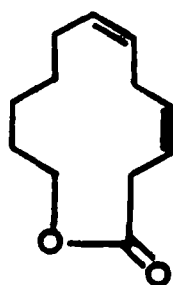
(Roman numeral notation as used by Pierce et al, 1984a, 1984b)

Of these three macrolide lactones, II and IV are chiral molecules, whilst III is achiral (Fig. 26).

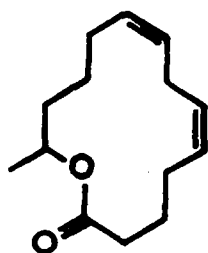
Synthetic samples of these materials (racemic mixtures in the case of II and IV) were supplied by Dr. A.M. Pierce, Simon Fraser University, B.C., Canada. They were quantified using GLC with methyl laurate as an external standard, and their purity was determined as:-
(R,S) II = 82%, III = 89%, (R,S) IV = 86%.



II (Z,Z)-3,6-dodecadien-11-olide



III (Z,Z)-3,6-dodecadienolide



IV (Z,Z)-5,8-tetradecadien-13-olide

FIG.26 Beetle-produced macrolide lactones identified by coupled GC - EAG.

To ensure that impurities present did not themselves cause an EAG response, each solution was tested using the GC-EAG technique. Traces showed that only the lactones caused an EAG response (Fig. 27).

Behavioural assays.

The level of attraction to aeration extracts and synthetic materials was measured using the 2-choice pitfall test (Chapter 4) with 20 individuals of mixed sex per test. The response to the aeration extract A1E1 was tested at 5 concentrations with 6 replicates at each concentration. Within the same randomised experiment, the response to a mixture of the 3 synthetic lactones at the concentrations found in A1E1 was also tested. GLC analysis showed the levels in A1E1 to be II = 2.78 ng/ul, III = 0.80 ng/ul, IV = 1.30 ng/ul , so they were tested in these amounts (mixture 1). However, it was not possible to determine whether the II and IV produced by beetles were optically pure or racemic mixtures. If beetles produce and respond to only one optical isomer, then to provide the same amount of active compound, twice the dose of the racemic mixture is needed. Therefore a mixture of 5.56 ng/ul II, 0.80 ng/ul III and 2.60 ng/ul IV was also tested (mixture 2).

Responses to single synthetic components and to a 1:1:1 mixture were also tested at various concentrations.

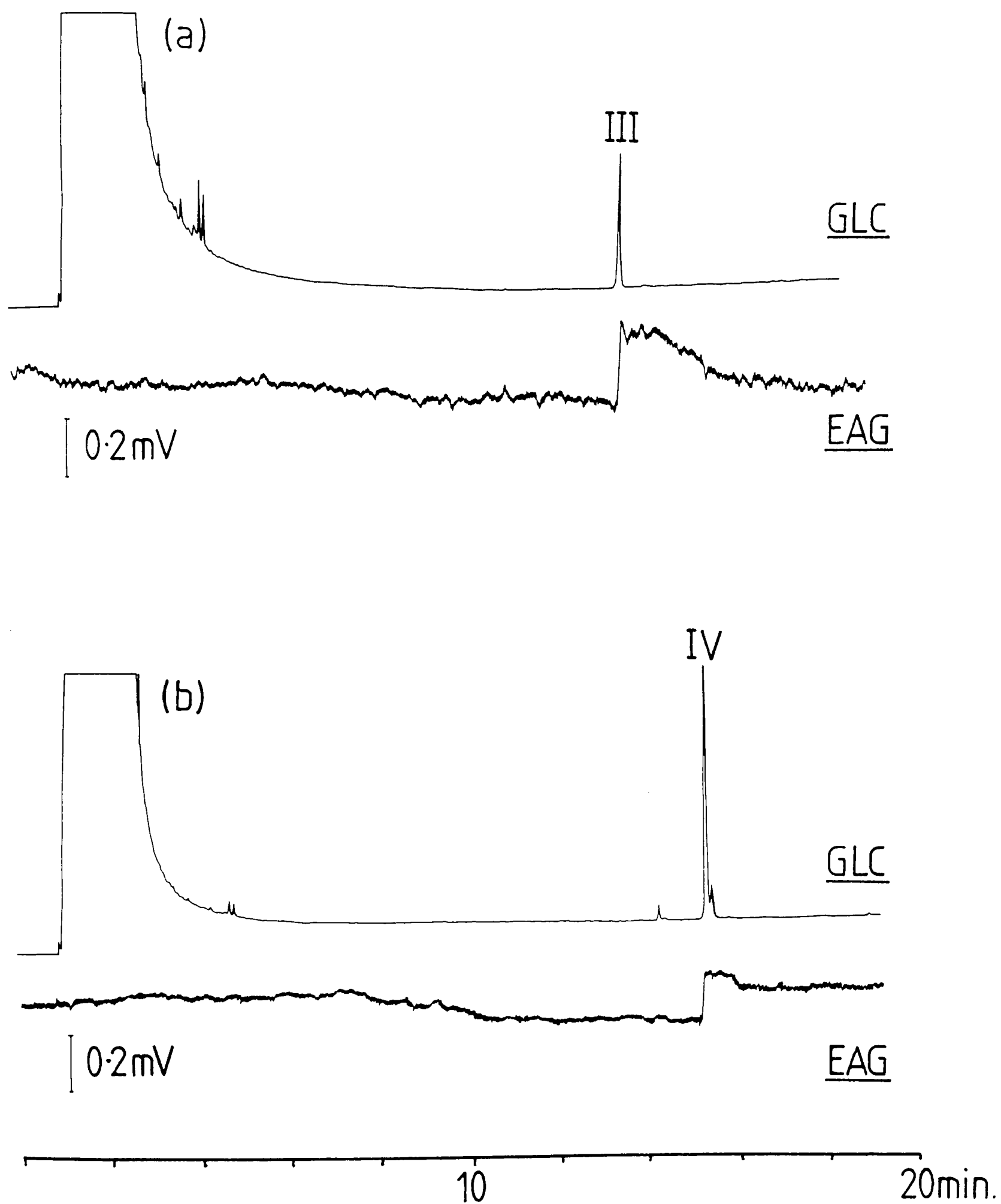


FIG.27 Coupled GC - EAG traces for synthetic pheromone components III (a) and IV (b) showing lack of EAG response to impurities.

RESULTS

GLC analysis of volatiles.

GLC analysis of the aeration extracts demonstrated over 200 volatile components produced by cultures of O. surinamensis adults on oats (Figs. 28,29), and the parallel aerations of oats alone (A3,A6) collected a similar number of volatiles (Fig. 29b). Careful comparison of retention times in the various extracts showed that although the ratios of volatiles varied between extracts, only 3 volatiles, with relatively long retention times, were collected exclusively from cultures containing beetles (peaks C,D and E in Figs. 28,29), and were thus beetle-produced.

EAG response to aeration extracts.

Analysis of the results showed that the EAG response of individuals to a standard dose (1.68 culture hours) of extract A4E2 (beetle plus oat volatiles) was significantly greater than the response to A3 (oat volatiles alone) ($F=55.5$, $P<0.001$. Table 3). However, there was no difference between the responses of the sexes to either extract ($F=0.1$ N.S.). Adult beetles were therefore shown to produce EAG-active volatiles.

Table 3. EAG response to aeration extracts.

		Extract	
		A3	A4E2
Sex	Male	88 +/- 28 uV	392 +/- 78 uV
	Female	75 +/- 20 uV	368 +/- 46 uV

Figures give mean EAG response +/- S.E. for 10 individuals.

Identification of EAG-active volatiles.

GC-EAG traces of the response to aeration extracts A1E1, A2E4 and A4E2 (beetle plus oat volatiles) each showed 3 major EAG responses, and 2 minor responses (Figs. 28, 29). Similar traces were obtained for both sexes.

The 3 major responses corresponded to the volatiles which only appeared in beetle aerations, whilst the materials causing the minor responses were also collected from aerations of oats alone.

GLC-MS analysis identified the 3 beetle-produced volatiles causing the EAG response as:-

Peak C= (Z,Z)-3,6-dodecadien-11-olide (II)

D= (Z,Z)-3,6-dodecadienolide (III)

E= (Z,Z)-5,8-tetradecadien-13-olide (IV)

The volatiles causing the minor EAG responses were identified as:-

Peak A= 1-octen-3-ol and 3-octanone. (These compounds eluted within seconds of each other and it is likely that their EAG responses overlapped).

Peak B= 1-nonanal.

GC-EAG traces of oat volatiles (A3) failed to identify any EAG active peaks, probably because the EAG response to the whole extract was low, not allowing responses to be distinguished from background drift in the EAG recording (Fig. 29b).

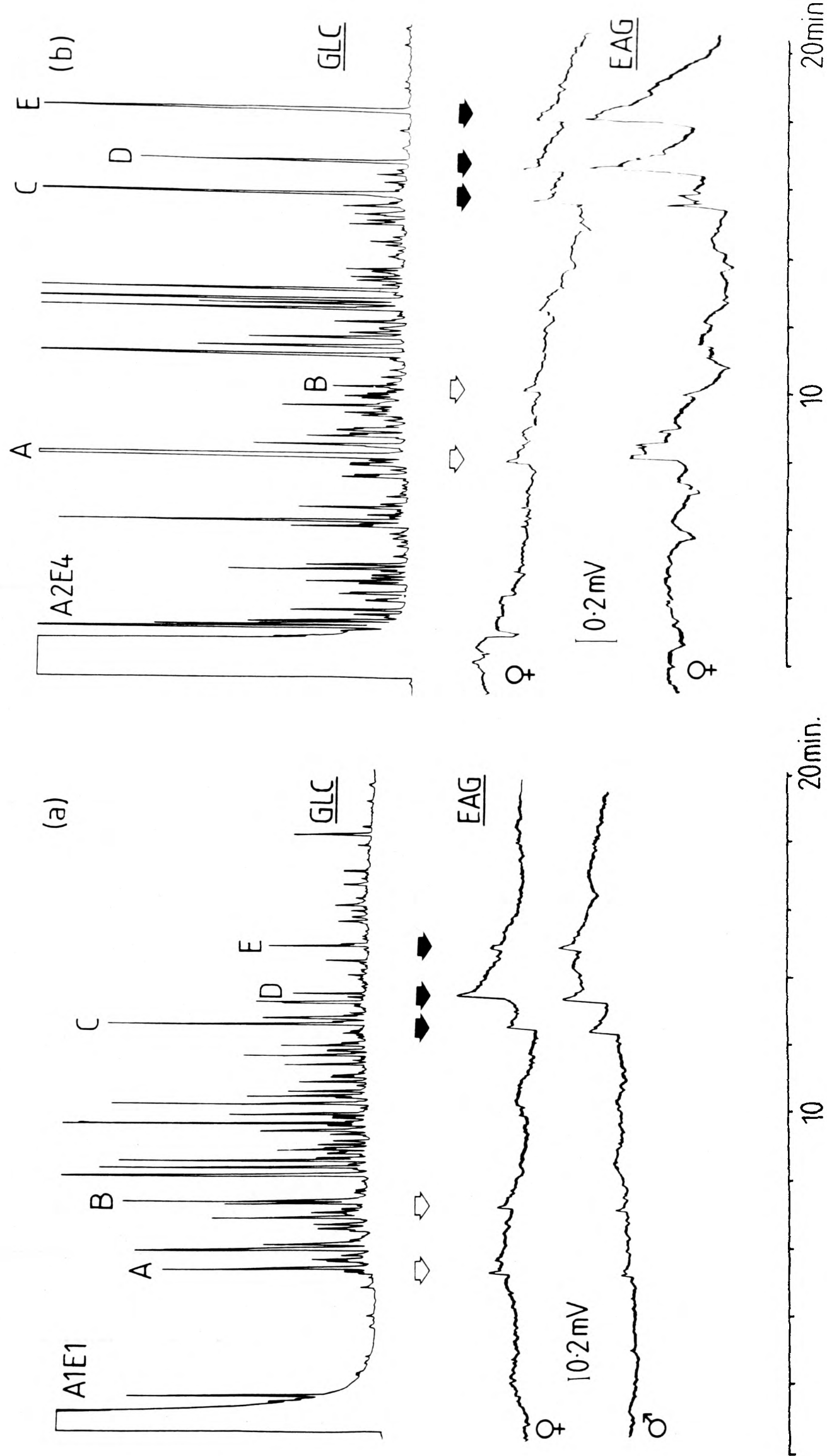


FIG.28 Coupled GC-EAG traces for (a) aeration extract A1E1 and (b) A2E4 each showing 3 EAG responses to beetle-produced volatiles (closed arrows) and 2 EAG responses to food-produced volatiles (open arrows).

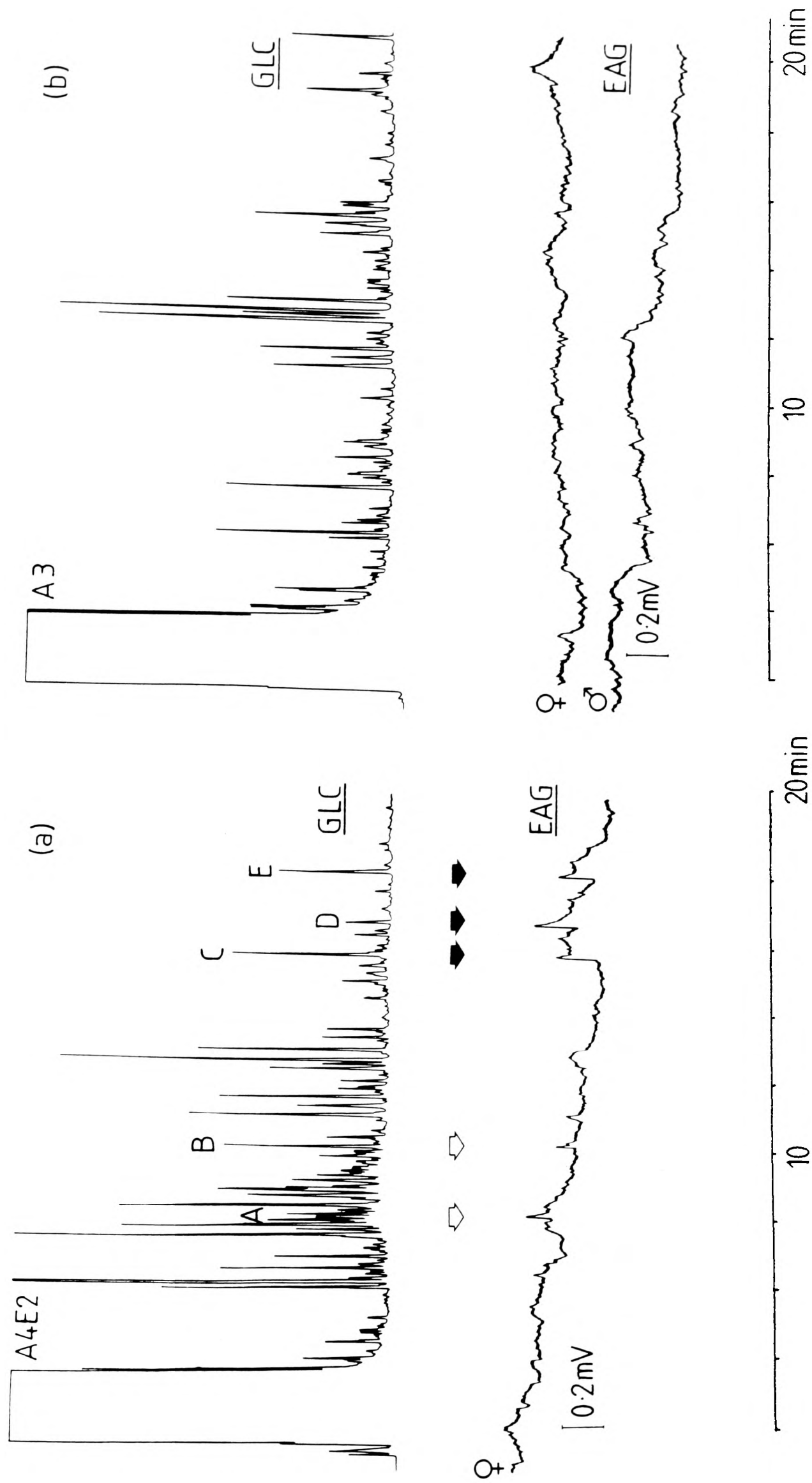


FIG.29. Coupled GC-EAG traces for (a) aeration extract A4E2 showing 3 EAG responses to beetle-produced volatiles (closed arrows) and 2 EAG responses to food-produced volatiles (open arrows), and (b) aeration extract A3 (food volatiles only) showing no consistent EAG responses.

Production rates and ratios of beetle-produced volatiles.

GLC traces of the aeration volatiles (Figs. 28, 29) showed that the amounts of the lactones II, III and IV varied widely between extracts, suggesting differing production rates. The maximum rate (assuming an average of 8000 beetles over the aeration period (4000 pheromone-producing males) and that all volatiles were collected) was II 0.10 ng/male/hr; III 0.03 ng/male/hr; IV 0.08 ng/male/hr. The ratio of lactones produced also varied from 2:1:1 to 8:1:2. The mean ratio of 8 extracts was 4.4 II: 1 III: 2 IV.

Behavioural assays.

The pitfall assay demonstrated that the aeration extract A1E1 was strongly attractive to adult beetles (Fig. 30). The equivalent mixture of synthetic lactones only accounted for about 27% of the response to the whole extract A1E1 (response to A1E1 = 70.9 $\pm$ 3.5 %; synthetic mixture 1 = 18.8 $\pm$ 2.4 %). However, if the amounts of II and IV were doubled to allow for the racemic nature of the synthetic material, the mixture of the lactones accounted for 87% of the response to the whole extract (response to synthetic mix 2 = 61.4 $\pm$ 4.3 %). Therefore, if chiral specificity in the response is assumed, most of the response to the extract A1E1 can be explained in terms of the 3 lactone components present.

The responses to the individual identified components showed different dose-response relationships (Fig. 31a). Lactone II was attractive by itself, although this effect decreased at higher concentrations. Lactone III, though less attractive, showed a similar trend, whilst lactone IV showed little activity except at the highest

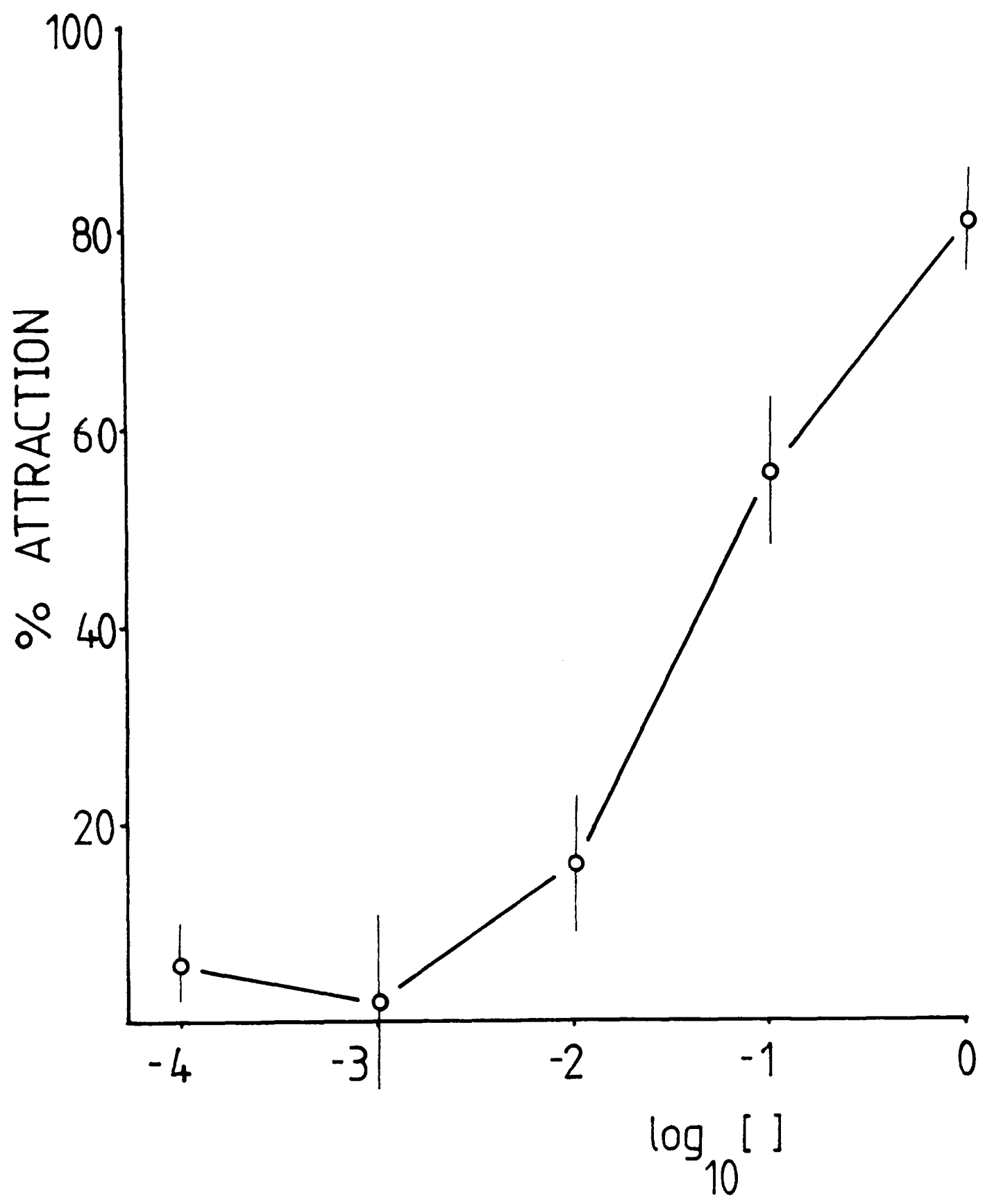


FIG.30 Behavioural response to aeration extract A1E1 (beetle plus food volatiles) in 2-choice pitfall bioassay. 5ul used of each concentration. 5ul of 10^0 concentration was equivalent to 1.68 aeration hours. Means $\pm$ SE, n=6.

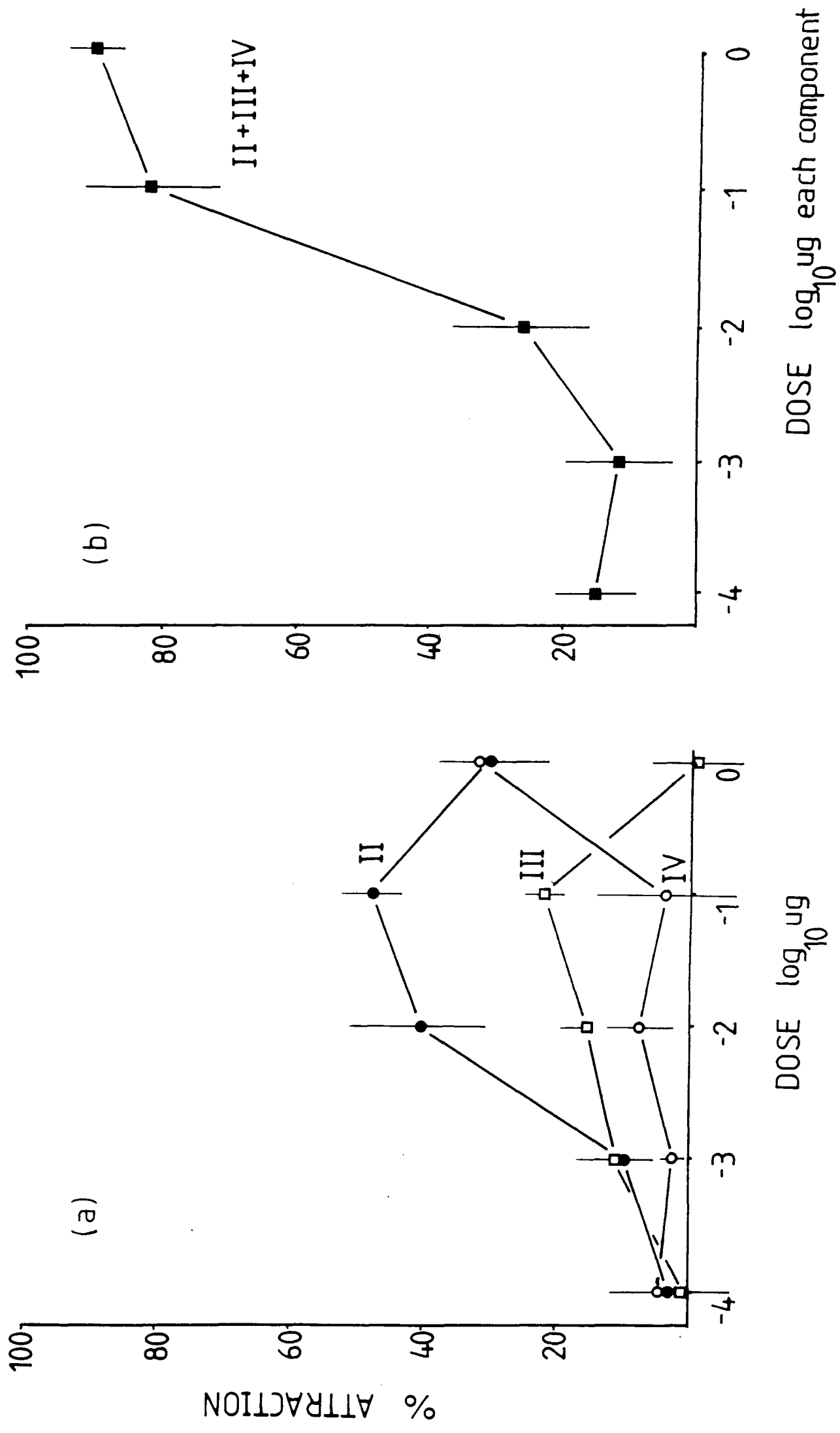


FIG.31 Behavioural response to synthetic pheromone components.
 (a) Response to individual components.
 (b) Response to a 1:1:1 mixture of components.
 Means $\pm$ SE, n=6.

concentration tested. However, the mixture of components was highly attractive (Fig. 31b), and subsequent examination of the individuals attracted to such a mixture showed that both sexes responded. This confirmed that these 3 components act as an aggregation pheromone in O. surinamensis .

DISCUSSION.

Examination of beetle-produced volatiles by coupled GC-EAG identified the same 3 components previously reported by Pierce et al. (1984a, 1985) using a totally different method, which suggests that both strains tested (Slough and Canadian strain) use the same compounds for intraspecific communication. Differences may occur, however, since two of the components are chiral compounds, and strains of the same species have been found to utilise different blends of optical isomers (e.g. Ips pini, Birch, 1984). The Canadian strain of O. surinamensis respond only to the R enantiomers of II and IV (A.M. Pierce, pers. comm.), but so far it has not been possible to identify the chirality of the lactones produced in this study, nor to test the response to chiral compounds. This will be attempted as suitable material becomes available.

The two strains did differ, however, in the ratio of the pheromone components produced. The Canadian strain produce a ratio of 1:1:3 whilst that measured above had a mean ratio of 4:1:2, demonstrating a different major component in the two strains. Such strain or population differences have been reported previously in several species (e.g. the turnip moth Agrotis segetum (Lofsted, 1984)). The above results also show ratio differences within a strain, however, since the ratio varied from 2:1:1 to 8:1:2. Such differences beg the question of whether different strains also respond specifically to their own ratio, and whether changes in blend ratio affect the response. This problem is addressed in Chapter 8.

The 3 other EAG-active compounds identified from the aeration extracts appeared to originate from the oats. 1-octen-3-ol and

3-octanone are known to be formed when grain is contaminated by mould (Kaminsky et al. ., 1975), whilst 1-nonanal has been previously identified in oat volatiles (Mikolajczak et al. ., 1984). In the same study, O. surinamensis was tested against 1-nonanal, but did not show any significant attraction even at the highest dose tested (100ug). In another study, however (O'Donnell et al. ., 1983), attraction to 1-nonanal was demonstrated, but using considerably higher dosages than those found in the above aeration extracts. Furthermore, recent preliminary experiments on the above 3 food-produced materials have shown little or no attraction at the concentrations found in the aeration extracts (J. Chambers- pers. comm).

The behavioural response demonstrated to the complex mixtures of volatiles collected therefore appears due to the 3 identified macrolide lactone components that form the aggregation pheromone. Detailed studies of the antennal and behavioural responses to these components are the subjects of the following three chapters.

CHAPTER 6.

AGGREGATION PHEROMONE: ANTENNAL SENSITIVITY AND SPECIFICITY.

INTRODUCTION.

Electroantennogram responses to pheromones have been investigated in a wide variety of species, especially from the Lepidoptera (for examples see Roelofs, 1984). With sex pheromones, a clear pattern emerges from such studies:- the sex producing the pheromone (normally female) gives smaller EAG responses than the responding sex (normally male) (e.g. White and Birch, 1987). The responding sex has both greater sensitivity i.e. a lower threshold, and a greater maximum EAG response amplitude, and as EAG amplitude has been related to the numbers of acceptor sites present (Payne, 1975; Dickens and Payne, 1977) this suggests sexual differences in the size of the pheromone acceptor site populations.

Aggregation pheromones attract both sexes (Borden, 1985) and consequently the pattern there is less clear. Although EAG responses can be recorded from the antennae of either sex, often clear sexual differences have still been found. In Tribolium castaneum, for example, female EAG responses to the male-produced aggregation pheromone were found to be greater than those of males (Levinson and Mori, 1983), suggesting differences in the acceptor site numbers. In other cases, however, such as Dendroctonus frontalis, no differences in antennal sensitivity to pheromone were found, despite observed differences in the behaviour (Payne, 1970, 1971, 1975). In yet other examples where more than one pheromone component occurs, such as the

boll weevil Anthonomus grandis , sexual differences in sensitivity have been found to some components, but not others (Dickens, 1984).

The aim of this study, therefore, was to investigate in detail the EAG response of O. surinamensis to its 3 component, male-produced aggregation pheromone (Chapter 5; Pierce et al ., 1984a). Many early studies of pheromone chemoreception investigated the responses to apparently single-component pheromones, but it is now clear that most insects use multicomponent pheromones (Silverstein and Young, 1976). As is the case in O. surinamensis , most beetle pheromones identified so far have been multicomponent blends (Tumlinson, 1985).

To detect each component of a multiple blend the receptor system must possess two features. Firstly, there must be a difference between components in the binding to, and transduction at the acceptor sites on the olfactory cells. Such acceptor sites could either be totally specific for single components, or could respond differentially to several components. Secondly, since the input to the brain consists of sequences of action potentials from individual receptor cells (rather than potentials from acceptor sites) (Mustaparta, 1984), the receptor cells must also respond differentially to each component. Since such differences are presumably conferred by the nature, position and density of acceptor sites on the receptor cell, each cell may respond to only one, or differentially to several components, achieving discrimination in the central nervous system (CNS) via labelled lines (Perkel and Bullock, 1968) or across fibre patterning (O'Connell, 1975) respectively.

Acceptor site specificity has been investigated previously in bark beetles (Payne and Dickens, 1976; Dickens and Payne, 1977; Light, 1980) and also in cockroaches (Nishino and Manabe, 1983, 1984, 1985) using a refinement of the EAG technique introduced by Payne and Dickens (1976) under the name of "differential adaptation". Using this technique in D. frontalis, a large proportion of acceptor sites were found to respond to several different pheromone components and host volatiles (Dickens and Payne, 1977). Indeed, one component, frontalin, was found to affect the acceptor sites for all other compounds tested, suggesting a lack of specific acceptor sites, and thus lack of labelled lines in the recognition of individual components. However, this technique does not enable the specificity of receptor cells to be determined, since even if specific acceptor sites for individual components are demonstrated, the specificity of a receptor cell will depend on whether the cell bears just one, or several different types of acceptor site. This requires recordings from single cells.

Results from single-cell recordings in both moths and beetles do not agree with the above results from D. frontalis. In many studies, cells responding to pheromones have been found to be specialised for a single component (Kaissling, 1979; Preisner, 1979; Den Otter, 1977; Mustaparta, 1979; Mustaparta et al., 1980; Wadhams et al., 1982), and this suggests that acceptor sites are specific for a single component, and that each receptor cell possesses acceptor sites (acceptors) of a single type. Such conflicting results could reflect species differences, or differences in the techniques used. The second part of this study, therefore, was to use both techniques on O. surinamensis to determine both acceptor site and receptor cell

specificities, and investigate how perception of the multicomponent pheromone in this species occurs.

MATERIALS AND METHODS.

Antennal sensitivity to pheromone components.

The sensitivity of male and female antennae to the 3 synthetic pheromone components (Chapter 5) was investigated using the EAG technique (Chapter 3). Each individual was presented with 9 concentrations of each component on a logarithmic scale, in ascending order of concentration and interspersed with blank (solvent only) and standard (10 ul of carob distillate) cartridges. The order of presentation of the pheromone components at each concentration was randomised. A total of 20 individuals, all 4 weeks old, were tested (10 per sex).

The results were normalised as a % of the standard response, to control for variability between preparations and within the life of a single preparation, and then analysed using a 2 level ANOVA to determine sexual differences and differences between the responses to the 3 pheromone components. An analysis was also performed on the raw (i.e. unnormalised) data for comparison, but both analyses gave similar results.

Acceptor site specificity: Differential saturation of the EAG response.

This technique was introduced by Payne and Dickens (1976) under the name of "differential adaptation" and has been used to determine receptor system specificity in bark beetle olfactory communication (Payne and Dickens, 1976; Dickens and Payne, 1977) and more recently in characterising the sex pheromone of Periplaneta brunnea (Nishino and Manabe, 1985). The term "differential saturation" more accurately

describes the technique, since at no time is there any evidence of adaptation in the receptors. Adaptation would be shown if, during continual stimulation with an odour, the amplitude of the EAG response fell to zero. However, in this technique, the response to the first odour remains at a maximum, i.e saturated, level prior to stimulation by the second odour (see Figs. 35a, 36).

Using this technique, the antenna was stimulated with a high concentration of one compound, sufficient to produce the maximum possible EAG response. During such stimulation, the antenna was assumed to be "saturated" by that compound, and all acceptor sites for the compound were assumed to be occupied. This could be tested by additional simultaneous stimulation with the same compound producing no further deflection in the EAG trace. After a short delay, a second compound (the "test" compound) was then presented simultaneously with the saturating compound. If this compound interacted only with the same acceptor sites as the saturating compound, there would be no further EAG deflection. However, if different acceptor sites existed for the test compound, there would be a further EAG response. If the two populations of acceptor sites were completely distinct, the amplitude of the EAG response to the test compound would be unaffected by the saturating compound, whilst any interaction of the saturating compound with the acceptor sites for the test compound would lead to a reduction in the EAG response. By reversing the order of presentation and calculating the % decrease in the EAG response to compounds when presented as a test, rather than as a saturating compound, it was possible to calculate the interaction of lactones II, III and IV and CD with each population of acceptor sites (Fig. 35c).

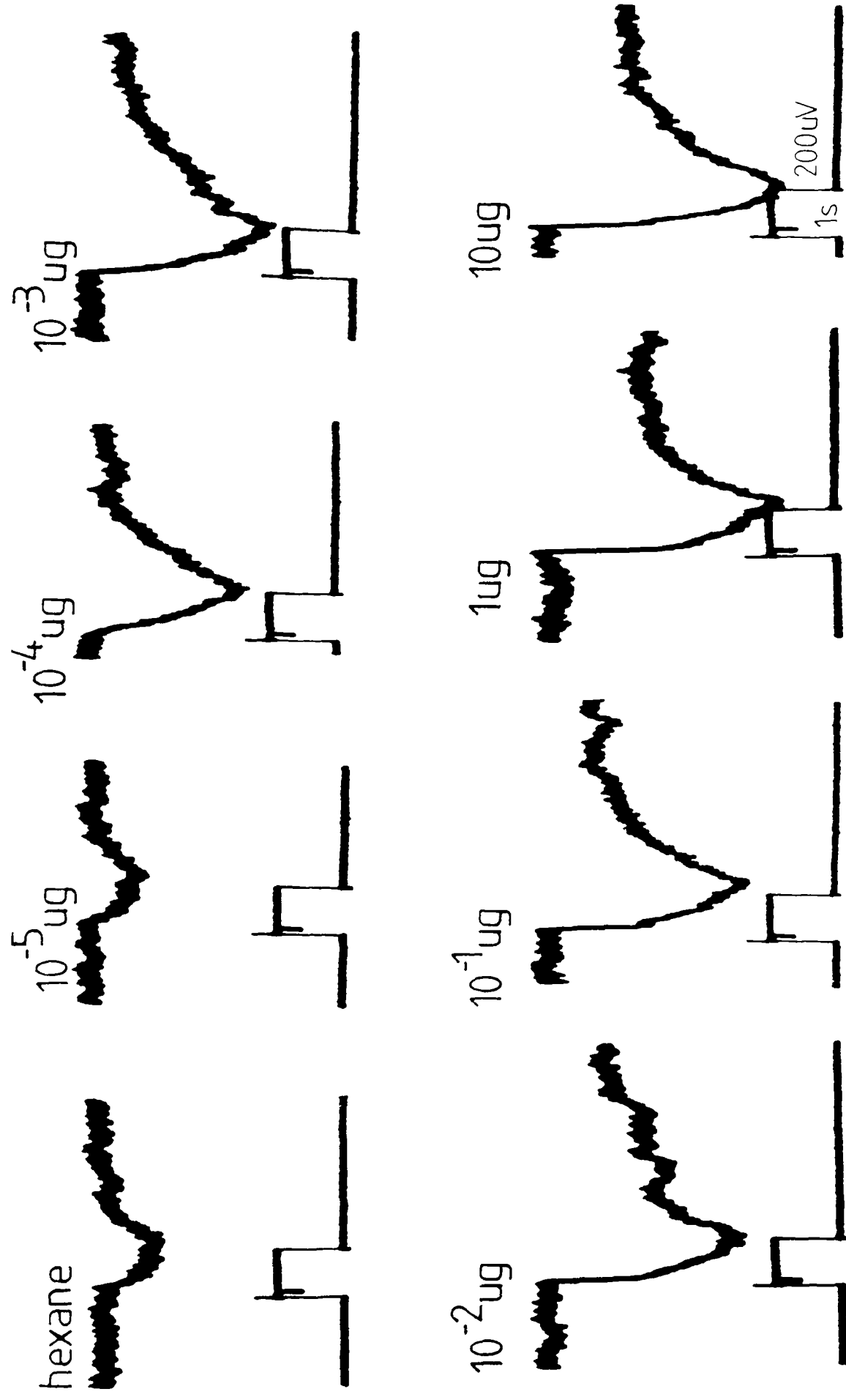


FIG.32 EAG response to pheromone component III. Sample traces from a single female insect.

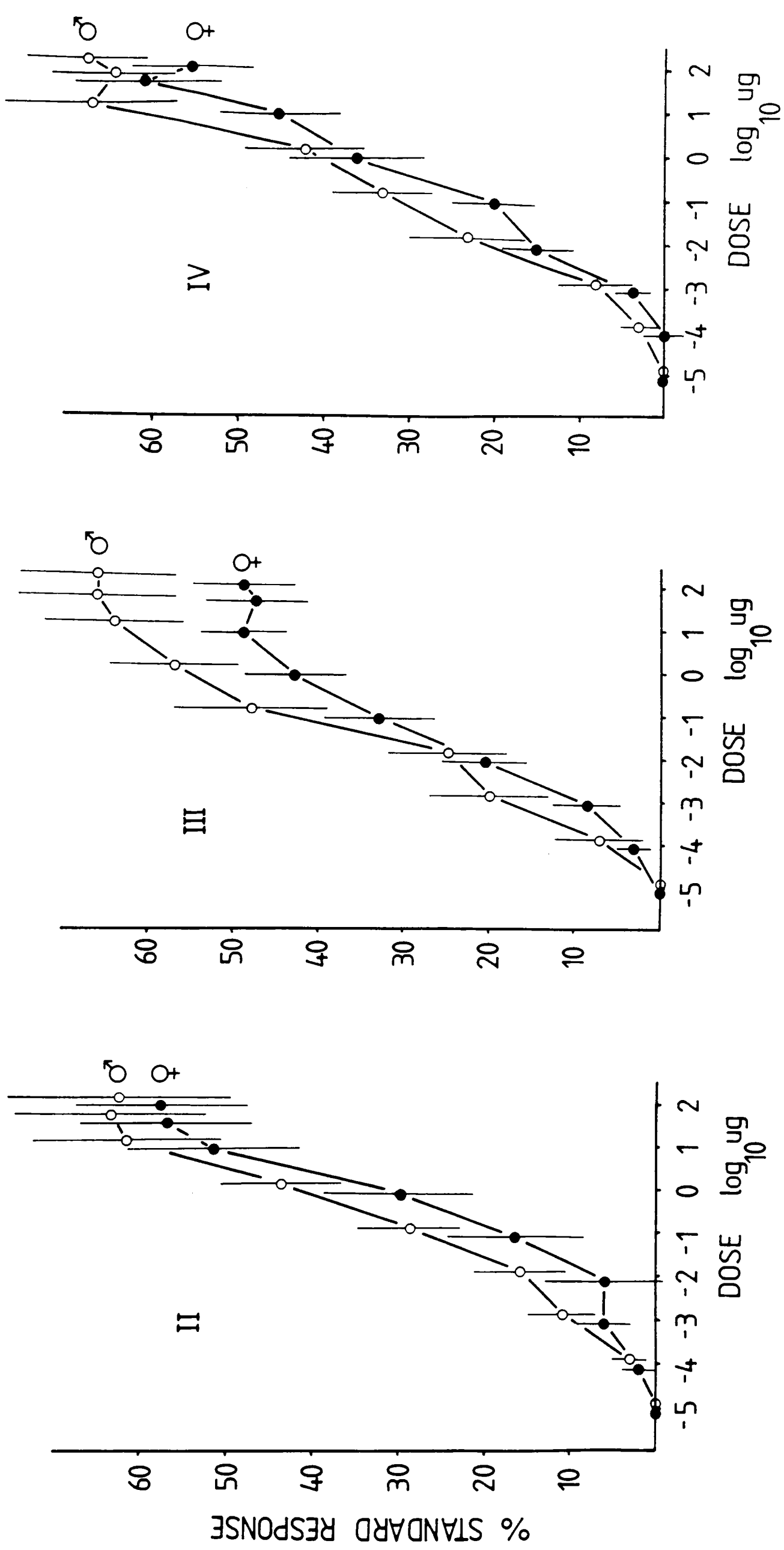


FIG.33 EAG response of Males (○) and Females (●) to synthetic pheromone components II, III and IV. (Means $\pm$ SE, n=10).

Individuals were set up for EAG recording as described above (Chapter 3), but with a modified odour delivery system (Fig. 34). The system used was similar to that described by Light (1980), presenting the test compound simultaneously with the saturating compound, rather than sequentially (Payne and Dickens, 1976). Prior to stimulation, the preparation was presented with clean air via both solenoid valves. Solenoid 1 (Fig. 34) was then activated, directing air via the saturating cartridge into the delivery tube. After 2 seconds, the timer activated solenoid 2 to give a 1 second pulse of air via the test cartridge, while solenoid 1 remained activated. Both solenoids were then inactivated, and the preparation allowed to recover in the clean air flow. Using this system the airflow over the antenna was kept constant at all times (2 l/min) and so responses due to the disturbance in the airflow were minimised (Fig. 35b).

Each preparation was presented with all possible paired combinations of lactones II, III and IV (10ug per cartridge) and carob distillate (50ul per cartridge) in random order, including successive stimulation by the same compound to ensure that saturation was complete. Due to the high doses used, preparations were allowed 10 mins to recover between tests. A total of 12 individuals were tested, 6 per sex. Differences between the levels of interaction between odours and acceptor site populations (Table 4) were tested by the non-parametric Wilcoxon matched pairs test (Siegel, 1956) and by the least significant difference method (LSD).

Receptor cell specificity: Single-cell recordings.

Preliminary recordings were carried out as described in Chapter

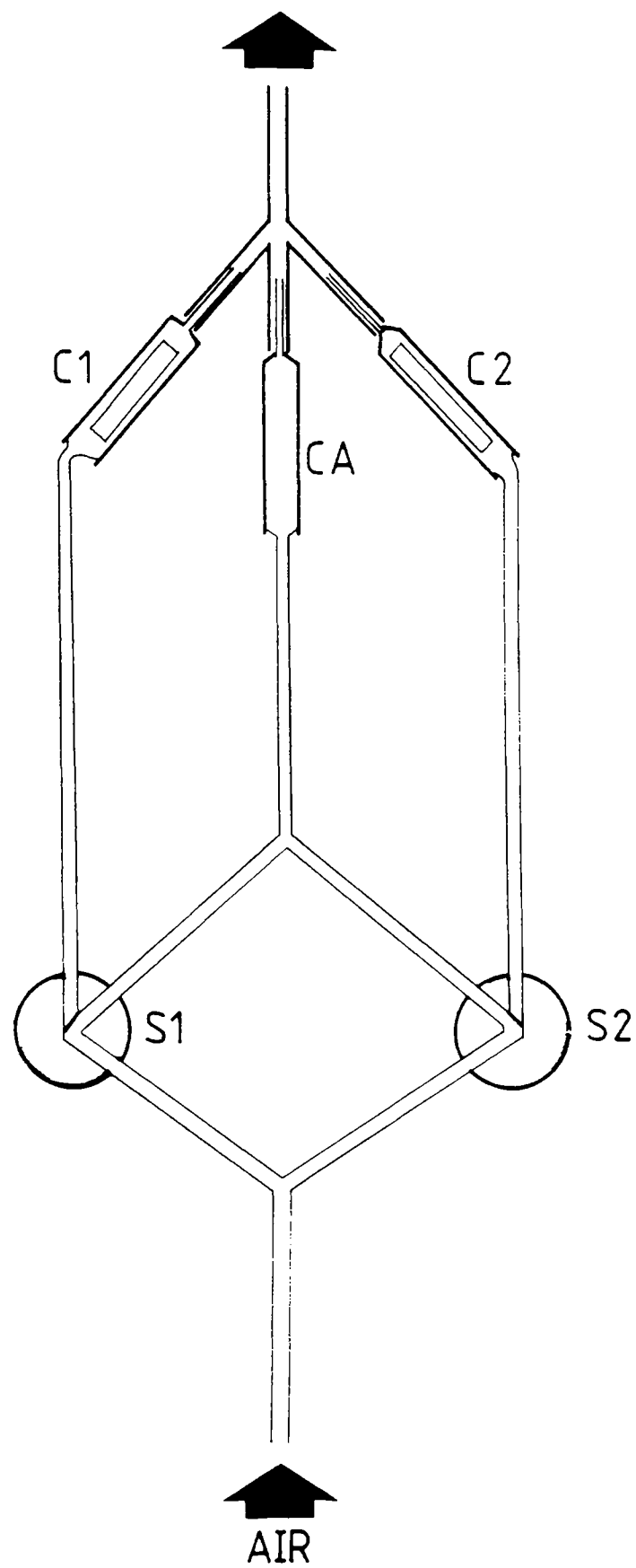


FIG.34 Odour delivery system for differential saturation experiment.

- S1 = Solenoid valve 1.
- S2 = " " 2.
- C1 = Cartridge containing saturating component.
- C2 = " " test " .
- CA = Clean air line.

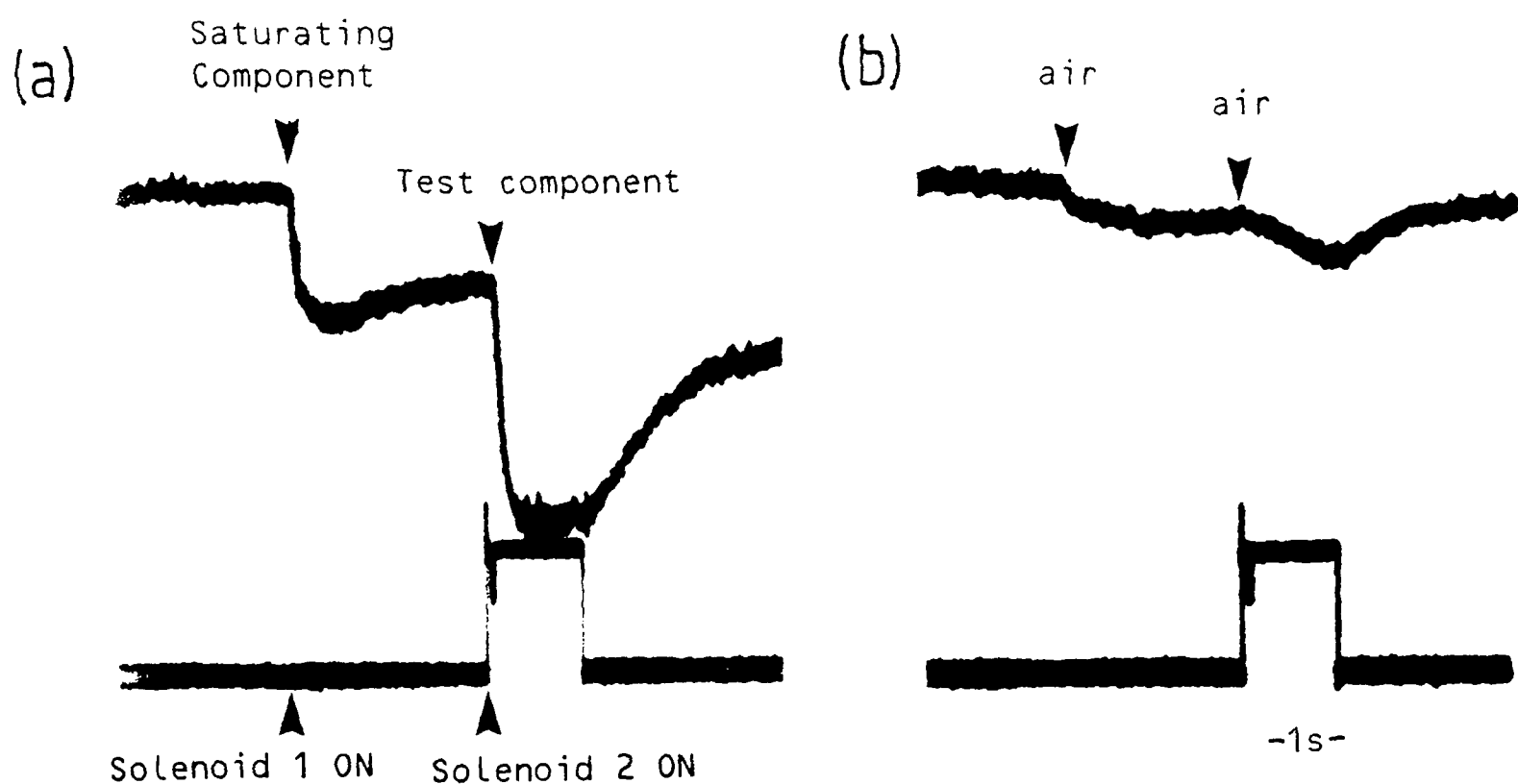
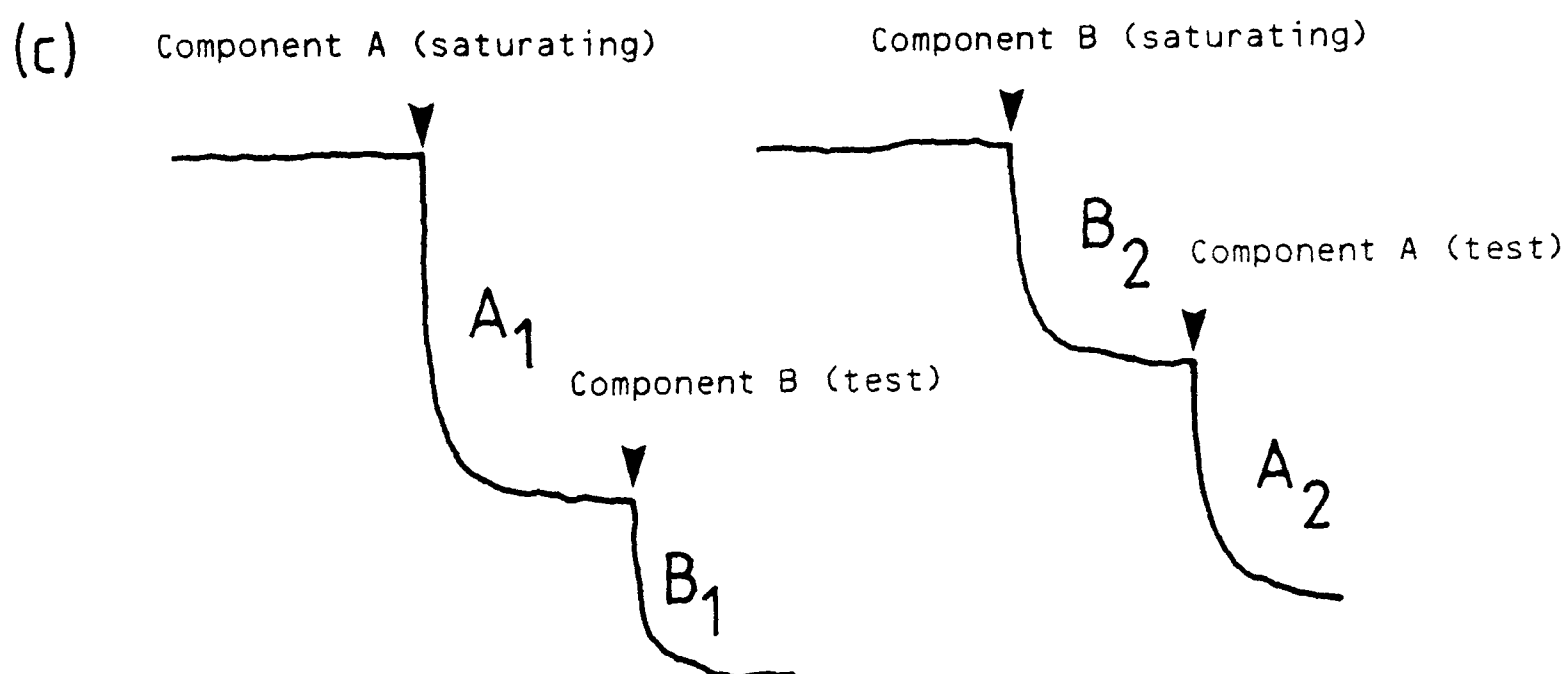


FIG.35 Differential saturation EAG traces for (a) two odours and (b) clean air i.e. control trace.



$$\% \text{ Interaction of Component A with acceptor sites for Component B} = \left[1 - \frac{B_1}{B_2} \right] \times 100$$

$$\% \text{ Interaction of Component B with acceptor sites for Component A} = \left[1 - \frac{A_2}{A_1} \right] \times 100$$

FIG.35 (c) Method used to calculate the interaction of odours and populations of acceptor sites. (From Dickens and Payne, 1977).

2. Cartridges containing lactones II, III and IV (1 ng), carob distillate (10ul), water (10ul) or solvent (10ul hexane) were presented, to investigate the specificity of single olfactory receptor cells. The activity of individual cells was distinguished by spike amplitude. A total of 9 cells were investigated from 2 individuals.

The results were analysed to determine whether each presentation caused a change in the firing rate from the baseline level.

RESULTS.

Antennal sensitivity to pheromone components.

Analysis of the results for all concentrations of the 3 lactones showed no differences between the responses of the 2 sexes using either the normalised ($F = 2.6$, N.S.) or raw EAG data ($F = 1.8$, N.S.). Comparison of the response curves for each sex (Fig. 33) showed similar thresholds, curve slopes and maximum EAG values.

The EAG responses to individual lactones did differ significantly ($F = 3.4$, $P < 0.05$ for normalised data; $F = 10.4$, $P < 0.001$ for raw data). Differences were greatest at lower dosages (Fig. 33), with III producing the largest responses, suggesting a lower threshold for this component. However, there were no significant differences between the maximum EAG responses to the 3 pheromone components ($F = 0.7$, N.S.)

Acceptor site specificity: Differential saturation of the EAG.

The decrease in the EAG response to each stimulus caused by pre-saturation with other compounds is shown in Figure 36 and Table 4. The overall pattern for both sexes was the same ($F = 0.5$, N.S.) and so the results from all individuals were combined.

The results show that the doses of chemicals used were sufficient to almost completely saturate the acceptor sites, since restimulation with the same compound (diagonal line of Table 4) reduced the subsequent EAG amplitude by more than 90% in each case.

The figures in Table 4 also represent the degree of interaction of each lactone with each other's acceptor site populations. Considerable interactions of some lactones with other acceptor site populations occurred. Lactone II, for example, interacted strongly

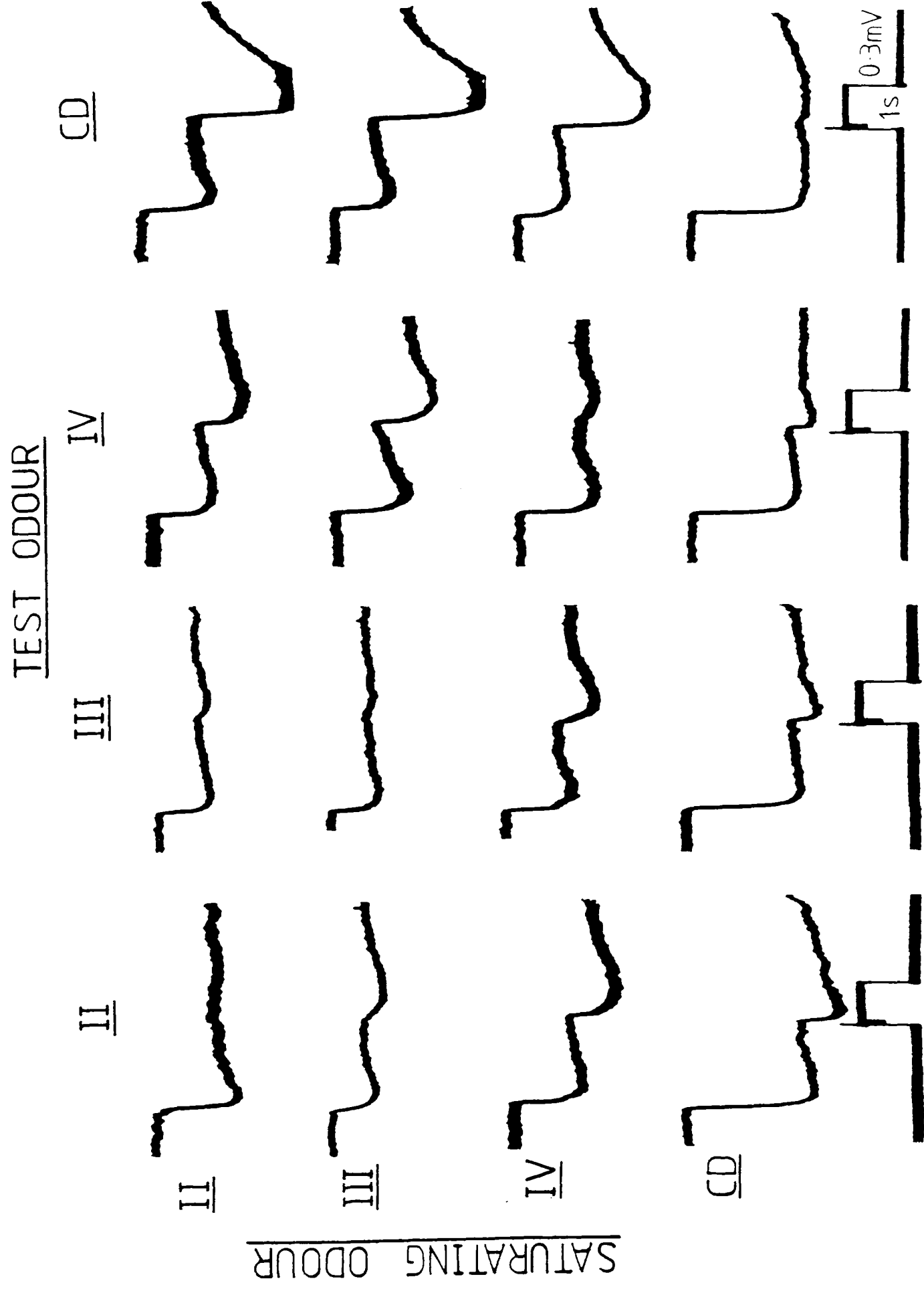


FIG.36 Differential saturation of the EAG response. Traces from one (female) individual.

TABLE 4. Differential Saturation of the EAG response.

TEST COMPONENT				
SATURATING COMPONENT				
II				
III				
IV				
CD				
II	98.3 +/-3.7 (a)	80.3 +/-4.8 (b)	38.1 +/-2.8 (fg)	26.7 +/-2.6 (hi)
III	72.3 +/-2.5 (bcd)	90.8 +/-2.1 (a)	33.6 +/-3.1 (g)	21.8 +/-2.4 (i)
IV	42.1 +/-3.3 (f)	50.4 +/-3.0 (e)	93.3 +/-2.1 (a)	30.0 +/-2.7 (gh)
CD	71.2 +/-4.1 (cd)	67.3 +/-3.8 (d)	76.0 +/-3.5 (bc)	91.8 +/-2.6 (a)

Figures give mean % reduction (+/-SE) of EAG response to Test component after exposure to the saturating component. Figures not followed by the same letter are significantly different (P<0.05, LSD test).
Means of 12 individuals, 6 males, 6 females.

with the acceptor sites for lactone III (80.3%) and vice versa (72.3%). Lactone IV reacted with the sites for lactones II and III and vice versa, but to a lesser extent. No lactones had much effect on the acceptor sites for carob distillate (II 26.7%, III 21.8%, IV 30.0%), but in the reversed order, carob distillate markedly affected the sites for the lactones (II 71.2%, III 67.3%, IV 75.9%).

Despite such interactions, however, the results do suggest that there were at least some acceptor sites specific to each lactone and to the components of carob distillate. For example, the interaction of III with the acceptor sites for II (72.3%) was significantly less than the interaction of II with its own sites (98.3%) ($P < 0.001$, see Table 4). This suggests that some of the II acceptor sites in this case were not filled by molecules of III, and were thus specific to II. A similar argument can be formulated for all other pairs of stimuli, to show that acceptor site specificity did exist to some extent.

Receptor cell specificity: Single-cell recordings.

Of the 9 cells tested, 3 responded to synthetic pheromone components, whilst 4 responded to the food odour carob distillate (Fig. 37a). All cells showed complete specificity at the concentrations used, and only responded to a single pheromone component or to the food odour (Fig. 37a,b). Unfortunately, it was not possible to investigate specificity at higher odour concentrations.

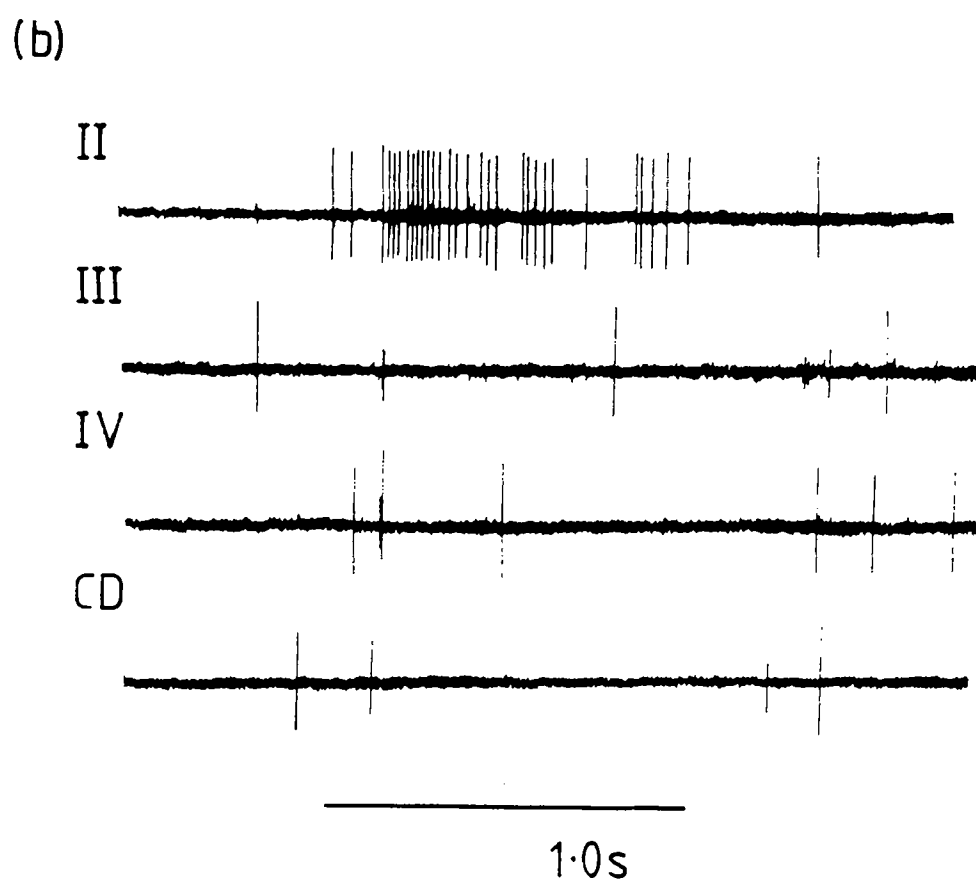
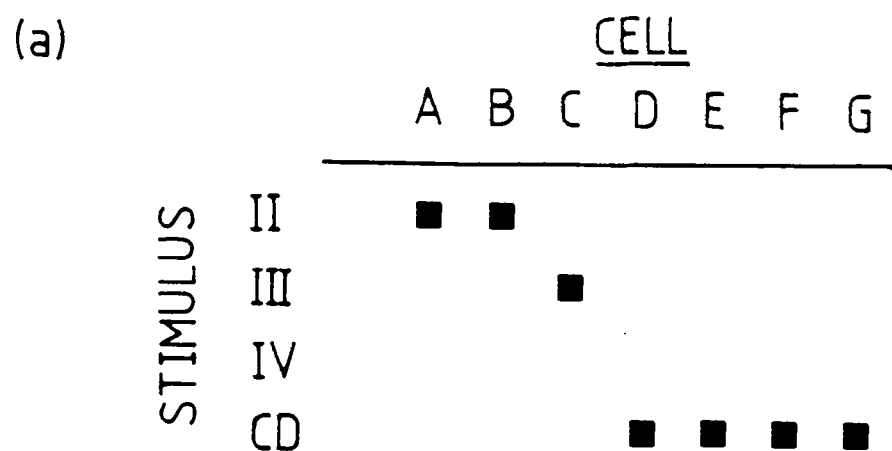


FIG.37 (a) Specificity of individual olfactory receptor cells. Table shows response spectra of 7 cells to synthetic pheromone components II, III and IV (1ng) and Carob Distillate (CD) (10ul).

(b) Response of a single olfactory cell to pheromone components II, III and IV, and Carob Distillate. (Note: Smaller spikes of a second cell also visible.)

DISCUSSION.

Antennal sensitivity.

Although the sex producing an aggregation pheromone is often less sensitive to one or more of the components than its partner (Dickens, 1986), this does not apply to O. surinamensis, since no sexual differences were found in either sensitivity or maximum EAG response to any of the 3 male-produced lactones. The differential saturation experiment also failed to show any sexual differences, suggesting that the observed lack of sexual difference was a real phenomenon, rather than due to high sample variability or small sample size.

The magnitude of the maximum EAG response has been suggested as a measure of the relative number of acceptor sites on the antenna for any given stimulus (Payne, 1975; Dickens and Payne, 1977). However, this assumes that all acceptor sites contribute equally to the EAG potential recorded, regardless of their type or position. Sites for different components may differ in their transductory amplification. Also, even if transduction at each acceptor site produces an equal local change in membrane permeability, distal sites on the olfactory dendrites might be expected to make a smaller contribution to the EAG response recorded in the antennal haemolymph than more proximal sites. The above correlation therefore also assumes a uniform distribution of different acceptor site types on the dendritic branches of the olfactory cells. Given such assumptions, however, the results imply that the number of acceptor sites on male and female antennae for any one pheromone component were approximately equal. Similarly, since for any individual the maximum EAG amplitude produced by each component

was roughly equal, acceptor site populations for each component appeared to be of equal size. The apparent greater sensitivity of antennae to lactone III may be due to the optical impurity of lactones used, rather than differences in the acceptor populations. If the acceptor sites respond only to one enantiomer, as often found in beetle antennae (e.g. Light, 1983a), and suggested by Pierce for O. surinamensis (pers. comm.) then the effective doses of II and IV used were lower than those of III, since only racemic mixtures of the former were available.

Even the maximum EAG responses to the pheromone components were low, however, compared to the response to the carob distillate standard, suggesting that the antenna of O. surinamensis bears more acceptor sites for food volatiles than for the aggregation pheromone components. Such a situation is uncommon, since the EAG responses to pheromone components are usually considerably larger. In the Douglas fir beetle Dendroctonus pseudotsugae, for example, male and female antennae are from 10 - 1000 times more sensitive to the aggregation pheromone components than to plant odours (Dickens, 1986). Some similar cases have been reported, however:- Male boll weevils (Anthonomus grandis) are as sensitive to cotton volatiles as to any pheromone components (Dickens, 1984). The results do suggest the relative importance of food volatiles to adult grain beetles.

Comparison with studies from other beetle species provide further interesting results. The maximum absolute (mV) EAG amplitude to any of the pheromone components was small (c. 0.3 mV) compared with most other species reported (Ips paraconfusus, Light, 1983a; Anobium

punctatum , White and Birch, 1987; Trogoderma granarium , Levinson et al ., 1978) where EAG amplitudes are typically in the order of 1-5 mV. This probably reflects the relatively low numbers of olfactory sensilla in O. surinamensis (White and Luke, 1986; Chapter 2; Light, 1980). Despite this, however, the sensitivity (i.e. threshold level) of the O. surinamensis antenna to the pheromone components compared favourably with these other species. Whilst such comparisons are tentative because of differences in the odour delivery systems used and the vapour pressures of the pheromone components, they suggest that unlike the maximum EAG amplitude, sensitivity is not solely a function of receptor population size, but will also depend on the sensitivity of individual receptors, and the arrangement of the receptors on the antenna.

Acceptor site and receptor cell specificity.

The differential saturation experiment showed varying degrees of acceptor site specificity, but conversely, as found in previous studies (Dickens and Payne, 1977; Light, 1980) considerable interactions between pheromone components and other acceptor site populations occurred. The degree of interaction reflected the chemical similarity between the relevant components, with large interactions where the molecular structures were most similar i.e. between lactones II and III. Pheromone components had little effect on the acceptor sites for the food odour but, unexpectedly, the food odour had a marked effect on the subsequent EAG response to all 3 pheromone components. Such asymmetrical interactions have been reported before and have been interpreted as evidence of differences in acceptor site specificity (Light, 1980). Here it may be due to the fact that the

food odour is a complex mixture, unlike the pheromone components. If a pheromone component affected one type of food odour acceptor site, others would be left unaffected, and so the pheromone would have little effect on the subsequent food odour response. Conversely, if one component of the food odour affected the pheromone component acceptor sites, this would cause a marked decrease in the subsequent pheromone EAG response.

As found in previous studies, the results for acceptor site specificity (differential saturation) appeared to contradict those for receptor cell specificity (single-cell recordings). The former suggested that pheromone components interact with each other's acceptor sites, and so receptor cells possessing such sites should respond to more than one pheromone component. However, this was not found; receptor cells responded to one component only, demonstrating clear specificity. The apparent discrepancy probably reflects the different stimulation levels used. Stimulation of single cells involved low odour concentrations, compared to very high levels used for the differential saturation tests. Single-cell recordings from Ips pini and Ips paraconfusus (Mustaparta et al. , 1980) and Scolytus scolytus and S. multistriatus (Wadhams et al. , 1982; Angst, 1981) showed that each cell tested was keyed to a specific pheromone component, but at high concentrations such specific cells also responded to other materials. This suggests that at such high concentrations, molecules will interact with acceptor sites to which they poorly fit, and this could explain the levels of interaction seen in the differential saturation tests.

The receptor cell specificity shown suggests that each receptor cell possesses acceptor sites for a single component, and that perception of the pheromone blend is achieved via a "labelled line" system, with separate cells keyed to individual components (Perkel and Bullock, 1968). Using such a system, O. surinamensis is theoretically able to detect changes in the pheromone blend ratio, which were found to occur between and within populations (Chapter 5). Experiments to show that beetles can discriminate behaviourally between the pheromone components were also conducted, and are described in Chapter 8.

CHAPTER 7.

AGGREGATION PHEROMONE: INTERSPECIFIC RESPONSES.

INTRODUCTION.

Pheromones, by definition, act intraspecifically (Karlson and Luscher, 1959), but such chemicals, once released into the environment, may affect the behaviour of any other sympatric species able to detect them. Thus pheromones may also act as allelochemicals (Whittaker and Feeny, 1971). Many examples of pheromones causing interspecific interactions have been recorded (for examples see Birch, 1984; Mustaparta, 1986) and fall into 2 general categories:- interspecific attraction or interruption.

Mutual attraction to the reciprocal aggregation pheromone has been shown to occur in Ips typographus and Dendroctonus micans (Tommeras et al.., 1984). The advantage to the emitter in such interactions is not clear, though the receiver is able to locate suitable host trees, since the 2 species occupy similar habitats. Such chemicals therefore probably act as kairomones (Brown et al.., 1970; Nordlund, 1981) since benefit accrues to the receiver. Further examples of pheromones acting kairomonally in interspecific attraction include their use by predators or parasites to locate their prey. Several species of the clerid beetle genus Thanasimus, for example, are predators of bark beetles, and are attracted by their aggregation pheromones (Bakke and Kvamme, 1981; Vite and Williamson, 1970; Dixon and Payne, 1979).

Interspecific interruption occurs when the pheromone of one species inhibits the attraction of another species to its own pheromone, and this has been clearly documented between the sympatric species Ips paraconfusus and Ips pini, where mutual interruption occurs (Birch and Wood, 1975; Birch et al., 1977, 1980; Light and Birch, 1979).

In all of the above examples of interspecific interactions, it has been possible to show electrophysiologically that the responding species possesses specific receptors for the pheromones of the other species (Tommeras et al., 1984; Tommeras, 1983). In the above case of mutual interruption, the sensitivity to the interspecific interruptant was greater than to the conspecific major pheromone component (Light and Birch, 1982). The possession of receptors for pheromone components from other sympatric species, therefore, would suggest the importance of interspecific interactions, although behavioural assays would be necessary to distinguish between interruptants and attractants.

Recently, aggregation pheromones have been identified from several other species of Silvanid and Cucujid beetles, often found with O. surinamensis in stored grain (Table 5, Pierce et al., 1984b; Millar et al., 1985a, 1985b; Borden et al., 1979; Wong et al., 1983). To date, however, only one instance of interspecific interaction has been reported:- volatiles collected from O. mercator frass and adults attracted O. surinamensis and vice versa (Pierce et al., 1981). As these closely related species are often found in the same habitats, other interspecific interactions are likely to

exist. This experiment was therefore conducted to determine the antennal response of the saw-toothed grain beetle to the pheromones of the other species, to indicate if, and with which species, such interactions are likely to occur.

MATERIALS AND METHODS.

Seven macrolide lactones reported from 5 species of grain beetles were tested for EAG activity. These were:-

(Z)-3-dodecen-11-olide (S)	(I)	Purity 99.0%
(Z,Z)-3,6-dodecadien-11-olide (R,S)	(II)	82.0%
(Z,Z)-3,6-dodecadienolide	(III)	89.0%
(Z,Z)-5,8-tetradecadien-13-olide (R,S)	(IV)	86.0%
4,8-dimethyl-(E,E)-4,8-decadienolide	(V)	99.7%
(Z)-3-dodecenolide	(VI)	97.0%
(Z)-5-tetradecen-13-olide (S)	(VII)	97.0%

Lactones I, V, VI and VII (I and VII as optically pure forms) were synthesised and supplied by Prof. K. Mori, Dept. of Agricultural Chemistry, Tokyo. Lactones II, III and IV were supplied by Dr. A.M. Pierce, Simon Fraser University, Canada. Each was quantified and tested for purity using capillary GLC.

Each lactone was presented at a single dose of 1ug per cartridge, in random order, to 10 individuals (5 per sex) and the amplitude of the EAG responses normalised as a % of a standard response (10ul carob distillate). Results were analysed by a 2 level ANOVA and differences between the individual lactones tested subsequently for significance using Fisher's protected LSD test.

RESULTS AND DISCUSSION.

At the concentration tested, the EAG amplitude was greatest to the conspecific pheromone components III, IV and II in that order (Fig.38). The response to lactones I, V and VI were all significantly greater than zero ($P < 0.01$), but were all lower than the responses to the pheromone components ($P < 0.001$). The response to lactone VII, however, did not differ from zero. No differences were found between the responses of the sexes to the range of lactones ($F = 0.1$, N.S.).

Clear specificity was therefore shown to the conspecific i.e. pheromone components II, III and IV. The responses to the allelochemicals were small (cf. Light and Birch, 1982) suggesting that few acceptor sites fitting such materials exist. Such small, but significant, responses to lactones I, V and VI may either have been due to interactions with the acceptor sites for the pheromone components (i.e. lack of specificity) or to the existence of small numbers of specific sites for these allelochemicals. Further studies of receptor or acceptor site specificity (Chapter 6) would be needed to distinguish between these causes.

The complete lack of response to lactone VII was of interest, since Pierce et al . (1984b) (Table 5) reported that it is produced in small amounts by O. surinamensis , although it was not detected in the aerations described below (Chapter 5). Lack of response supports the view of Pierce et al . that it does not act as a pheromone in this species. It could act as a kairomone, and attract other species to aggregations of O. surinamensis , but the benefit to the emitter in such a situation is far from clear. Alternatively, it could

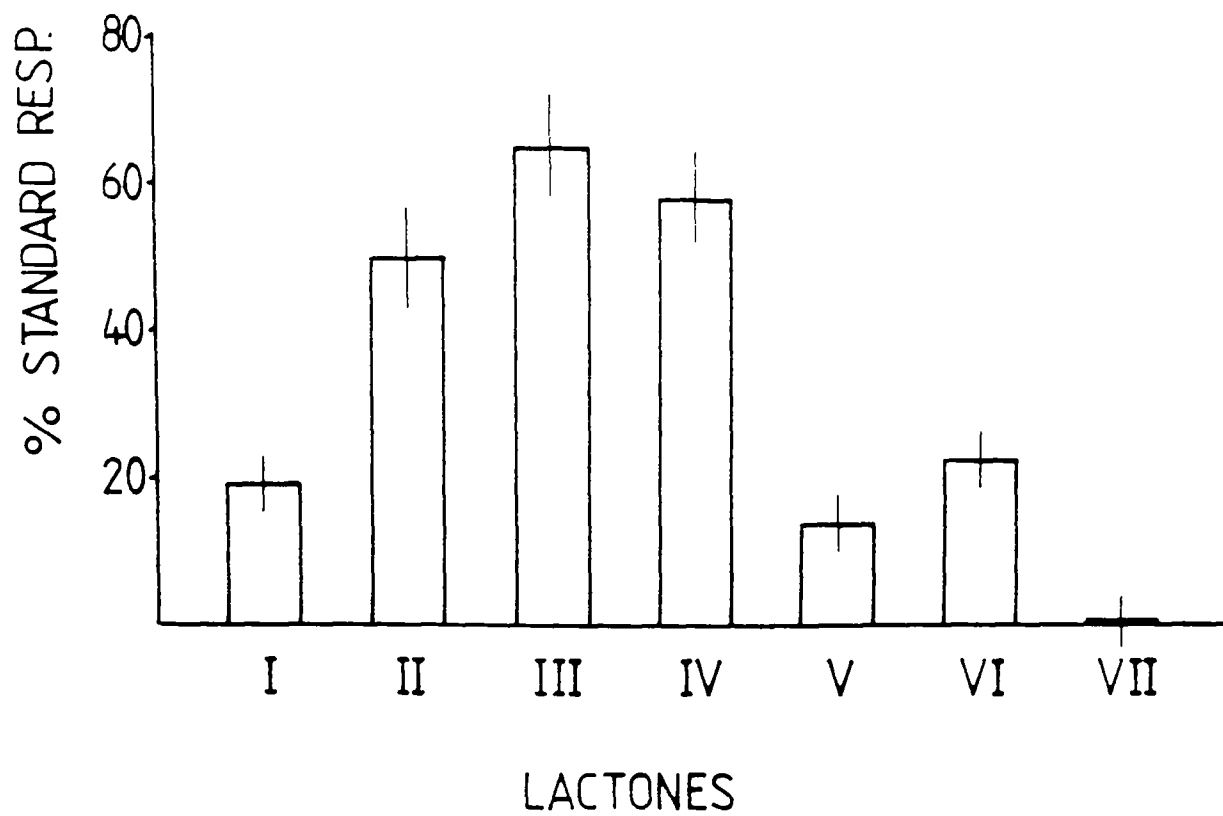


FIG.38. EAG response of *O. surinamensis* to 1ug of macrolide lactones I - VII. (Mean $\pm$ SE, n=10.)

Table 5. Distribution of macrolide lactones in *Cryptolestes* and *Oryzaephilus* species.

Species	I	II	III	IV	V	VI	VII
<i>C. ferrugineus</i>	P	+	-	+	P	-	+
<i>C. pusillus</i>	-	-	+	-	-	P	P
<i>C. turcicus</i>	-	-	-	P	-	-	P
<i>O. mercator</i>	P	P	+	+	-	-	+
<i>O. surinamensis</i>	-	P	P	P	-	-	+

P = Pheromone, + = produced by males in trace amounts, - = absent.

From Pierce *et al.* (1984b).

represent a vestigial compound which acted as a pheromone in an ancestral species shared with present species that still utilise this component in their pheromone blend.

The apparently small numbers of receptor sites for allelochemicals suggest that there are weak, if any, interspecific interactions. Interspecific attraction may occur by another mechanism, however, the use of common pheromone components by different species. Although each species uses a distinct mixture, several lactones have been detected in more than one species (Table 5), and this may explain the interspecific attraction observed between O. mercator and O. surinamensis (Pierce et al.., 1981). However, studies of chirality have shown that different enantiomers occur in different species (e.g. S-I in C. ferrugineus , and R-I in O. mercator ; Wong et al.., 1983; Pierce et al.., 1984a) and so species specificity may not only be achieved by the distinct mixture of the pheromone blend, but also by the unique identity of the blend components themselves.

This work therefore shows that O. surinamensis is considerably more sensitive to its own pheromone than to those of other grain beetles, which should lead to single- rather than mixed-species aggregations.

CHAPTER 8.

AGGREGATION PHEROMONE: BEHAVIOURAL RESPONSES TO INDIVIDUAL COMPONENTS AND BLENDS.

INTRODUCTION.

Chemical communication in O. surinamensis utilises beetle-produced volatiles which have been described as a multicomponent aggregation pheromone (Chapter 5; Pierce et al ., 1984a). This classification, although accurate, belies the complex mode of action of such chemicals.

Aggregation pheromones have been defined as "substances produced by members of either or both sexes, that induce members of both sex to aggregate" (Borden, 1985). Several functions have been ascribed to such aggregations. In the aposematically coloured beetle Lycus loripes , pheromone mediated aggregations provide mutual protection against predation (Eisner and Kafatos, 1962), whilst amongst bark beetles that attack and kill previously healthy trees, large aggregations are necessary to overcome the host tree's defences (Birch, 1984). It has also been suggested that aggregation pheromones have been evolved as indicators to conspecific individuals of a suitable food source or habitat (Shorey, 1973; Borden, 1977), although it is difficult to explain the advantage to the emitter of attracting potential competitors to a food source, unless group feeding or conditioning of the habitat occurs.

Since in many examples only one sex produces the aggregation

pheromone, it is likely that such pheromones have a sexual function related to mate location or courtship. In such cases, although both sexes respond, the pheromone might be expected to attract primarily the non-producing sex, and thus act in part as a sex pheromone. In the bark beetle genus Dendroctonus, for example, colonising females release myrcene and exo-brevicomin which preferentially attract males (Wood, 1982). In contrast, field tests with several species of Ips have demonstrated that more females than males are attracted to the male-produced aggregation pheromone (Wood, 1972; Birch and Light, 1977; Lanier and Burkholder, 1974; Lanier and Wood, 1975). Similarly, amongst stored-product insects, female red flour beetles Tribolium castaneum aggregate more than males in response to the male-produced chemical (Levinson and Mori, 1983).

Pierce and co-workers (1984a) reported that the aggregation pheromone of O. surinamensis was male-produced, and that both sexes were attracted. This study aimed to investigate further the separate responses of the sexes.

The pheromone produced in the saw-toothed grain beetle consists of 3 components (Chapter 5), and such multicomponent systems are typical of beetles (Tumlinson, 1985) and insects in general (Silverstein and Young, 1976). Such systems may operate in two ways:- either each component causes a separate behavioural response (and thus would constitute separate pheromones), or all components are required as a specific blend before a response can be elicited (i.e. a multicomponent pheromone).

Most work on pheromone blends has been conducted on the sex

pheromones of moths. Several studies have suggested that different components affect separate behaviours in the approach of males to the calling females (Baker et al ., 1976; Bradshaw et al ., 1983). However, recent work has shown that all of the components are needed, even the minor ones, for maximum sensitivity to the pheromone, and that the blend acts as an integral unit (Linn et al ., 1984, 1986; Baker and Carde, 1979).

In many such blends, the ratio of components confers species-specificity on the pheromone, since many closely related species use the same components (Roelofs and Brown, 1982). In O. surinamensis , however, the ratio of components appears to be variable, both within and between populations (Chapter 5). The following study therefore attempted to investigate the role of individual components and mixtures in the attraction of conspecifics, and to determine the effect of changes in the blend ratio on the level of response.

METHODS AND MATERIALS.

i. Sexual differences in attraction to the aggregation pheromone.

Sexual differences were determined by testing the attraction of males and females, separately, to a mixture of synthetic pheromone components in the natural ratio produced by males (Chapter 5).

Large numbers of insects were cultured, removed on emergence as adults, sexed (Halstead, 1963) and kept in single-sex cultures at equal densities for 4 weeks before use. Insects were removed from culture jars 24h prior to testing, and kept unfed in 5cm x 2.5cm glass tubes. Tests were carried out using the 2-choice pitfall assay described above (Chapter 4), with 20 insects per test.

The % attraction to the synthetic pheromone components II, III and IV in the ratio of 8:1:4 (using racemic mixtures of II and IV) was determined over a range of 5 dosages on a logarithmic scale, with 10 replicates of each sex at each dose. The experiment was conducted as a fully randomised design and the results tested by analysis of variance to determine any sexual differences in the responses.

Although the above experiment would identify sexual differences in attraction to the aggregation pheromone, it did not control for differences in culture conditions prior to testing. As males produce the pheromone, females would not have been exposed to pheromone prior to the tests, in contrast to the males, and this could be responsible for any observed differences in their responses. To examine this possibility, therefore, a second experiment was conducted in which insects were removed from culture at emergence, sexed, and both single

and mixed-sex cultures set up. These were tested as before after 4 weeks, at a single dose of the synthetic pheromone mixture (1.0 ug = c.2780bh). After testing, the insects in the mixed-sex assays were sexed to determine the % response of each sex in each test. Thus, for the mixed-sex insects, the culture and test conditions were identical for both sexes, and so could not affect the behavioural response.

Results were analysed using a 2-way ANOVA to identify any effects of sex and culture type on the attraction to the pheromone mixture.

ii. Attraction to individual and multiple pheromone components.

For both this and the subsequent experiment, insects were raised as mixed-sex cultures, used when 4 weeks old and sexed on the day prior to use. Using the 2-choice assay, each sex was tested for its response to individual pheromone components, binary mixtures and a mixture of all 3 components. To maintain the correct ratio, the amounts of each component used were 0.8 ug II, 0.1 ug III and 0.4 ug IV.

iii. Effect of blend ratio on attraction to aggregation pheromone.

To test the effect of blend ratio, each sex was tested against a variety of blends. The blends used were 8:1:4 (produced ratio) and all other permutations of this combination i.e. 8:4:1, 4:8:1 etc., plus 4.3:4.3:4.3 (hereafter referred to as 4:4:4) and 3:1.5:9 (Canadian ratio - see Chapter 5). The same total amount of material was used in each test (1.3 ug), using racemic mixtures of lactones II and IV. (The optical purity of II and IV produced by beetles is not known. If beetles produce and respond to only one enantiomer, this could lead to

slightly different total amounts of active components in each of the blends tested. However, such possible differences would be small, and would not affect the male/female response ratio to any blend.)

Both this and the previous experiment involved fully randomised designs, with 5 replicates of each sex for each test mixture. Results were analysed using ANOVA and subsequent regression analysis.

RESULTS.

1 Sexual differences in attraction to the aggregation pheromone.

Analysis of the pitfall assay results (Fig.39) showed a highly significant difference between the sexes in their response to the synthetic pheromone mixture ($F=23.9$, $P<0.001$), with females showing the greater response at all doses above the threshold level of c. 1ng. The subsequent experiment on the effect of sex and culture type on the response also showed that more females than males were attracted to the pheromone ($F=46.5$, $P<0.001$) (Table 6).

Table 6. Effect of sex and culture type on attraction to synthetic aggregation pheromone.

	Male	Female
Single-sex culture	31.5 +/- 7.7	83.7 +/- 3.8
Mixed-sex culture	52.3 +/- 5.8	76.0 +/- 3.1

Figures give % attraction +/- S.E. $n=10$ for single-sex tests, $n=20$ for mixed-sex tests, giving equal numbers of each class tested.

Culture type was not significant as a main effect ($F=1.4$, $P=0.25$), but there was a significant interaction between sex and culture type ($F=6.6$, $P=0.01$). Inspection of Table 6 shows that fewer males were attracted in the single-sex tests than in the multiple-sex tests, whilst in females there was a slight bias in the opposite direction.

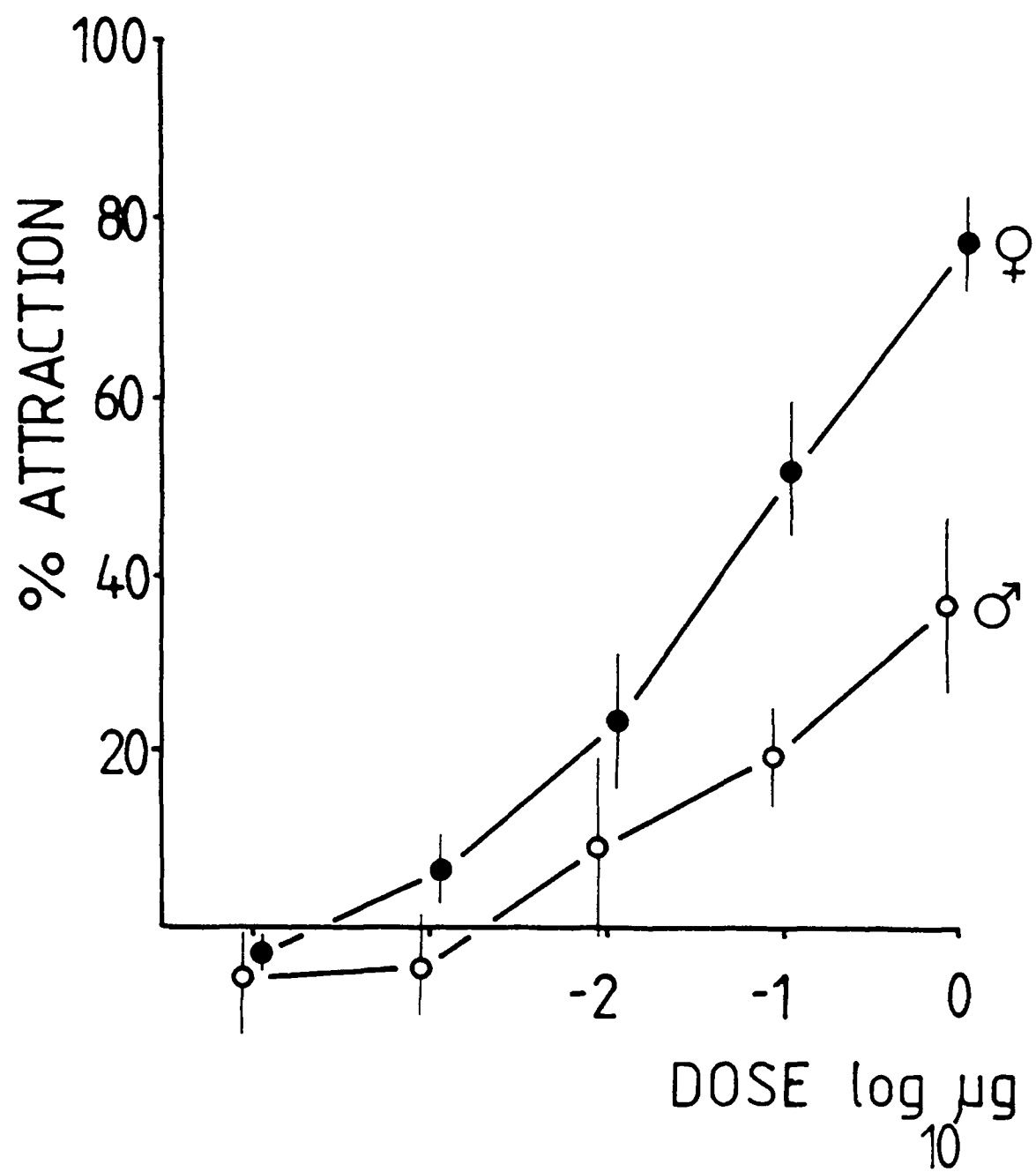


FIG.39 Attraction of males (○) and females (●) to the synthetic pheromone mixture, (8:1:4 blend ratio), in 2-choice pitfall bioassay. (20 insects/test, mean $\pm$ SE, $n=10$).

ii. Attraction to individual and multiple pheromone components.

The results broadly agreed with those previously obtained in mixed-sex tests (Chapter 5), with components II and III causing limited attraction, either singly or together (Fig.40a). When used alone, IV produced no significant effect, but caused a marked increase in the attraction of both sexes when added to III, and an increase in the attraction of females when added to II. In females, the largest response was produced by the presence of all 3 components, whilst in males, a 2 component mixture of III and IV was as effective as the complete mix.

Insect sex was a highly significant main effect on the behavioural response ($F=16.2$, $P<0.001$), with a similarly significant interaction between sex and the different mixtures tested ($F=5.2$, $P<0.001$). Examination of this interaction (Fig.40a) showed that although overall more females than males were attracted, significant differences between the sexes were only observed in response to mixtures containing component II. The results therefore suggest that this pheromone component had little or no effect on male beetles, since its presence in a mixture did not affect their response.

iii. Effect of blend ratio on attraction to aggregation pheromone.

The analysis of variance showed that both blend ratio and sex of the test insects were highly significant as main effects on the level of attraction (Blend: $F=4.4$, $P<0.001$; Sex: $F=25.0$, $P<0.001$) (Fig.40b), again with more females than males responding to the pheromone mixtures.

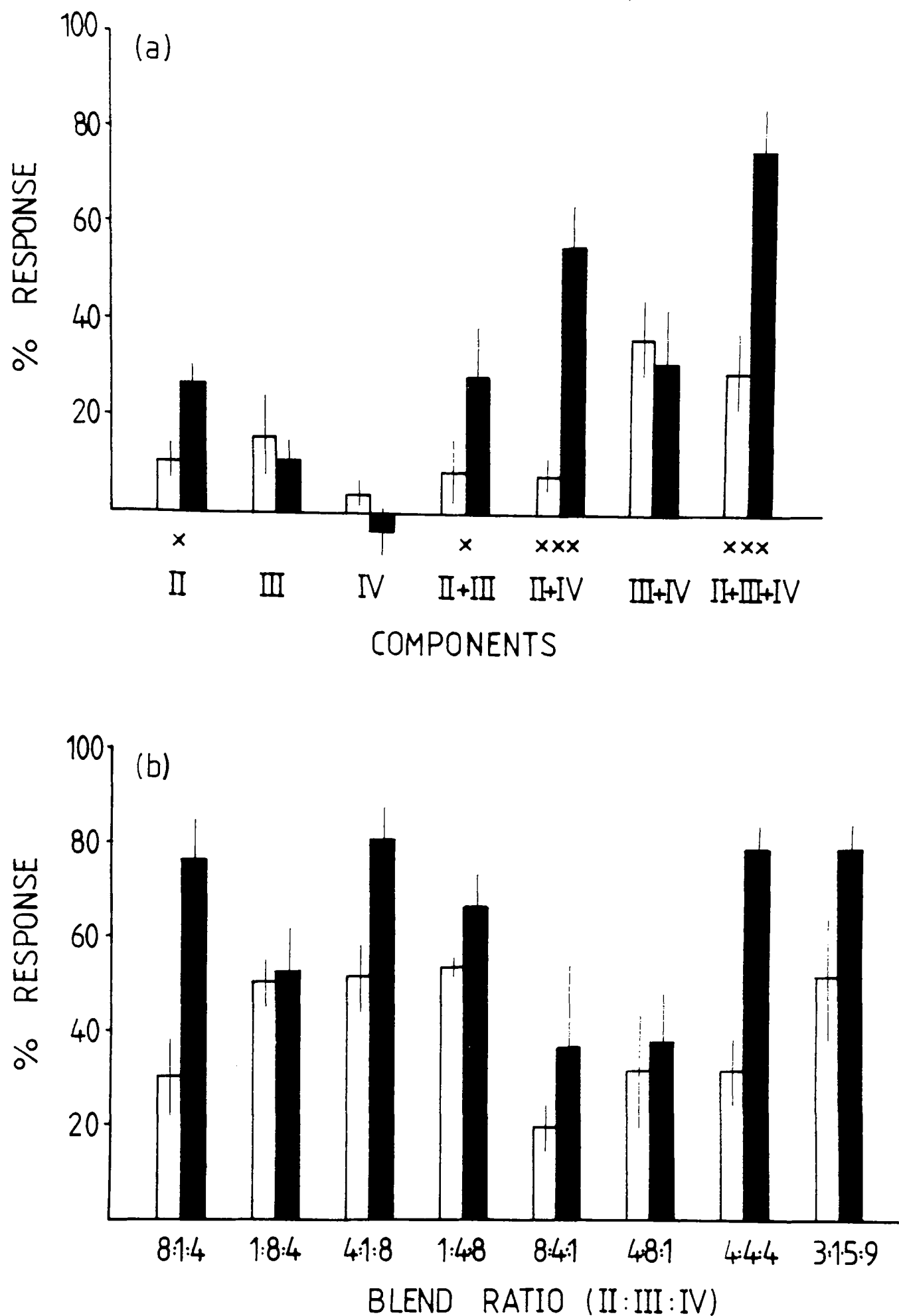


FIG. 40 (a) Attraction of males (open blocks) and females (closed blocks) to individual pheromone components and mixtures in 2-choice pitfall bioassay. (mean $\pm$ SE, $n=5$).

(b) Effect of blend ratio on attraction of males (open blocks) and females (closed blocks) in 2-choice pitfall bioassay. (20 insects/test, mean $\pm$ SE, $n=5$).

The results have been replotted in Figure 41, from which two trends can be detected. Firstly, the level of attraction of both sexes was positively correlated with the amount of component IV in the blend (i.e. vertical axis of Fig.41). Regression analysis confirmed this relationship:- regression of % attraction v amount of component IV gave linear relationships with significant positive slopes for both males (slope $b=0.35$ %/ug; $F=12.7$, $P<0.001$) (Fig.42a) and females (slope $b=0.45$ %/ug; $F=12.25$, $P<0.001$) (Fig.42b). Neither of the other lactones gave significant positive regression slopes.

Secondly, the ratio of male to female response (M/F) increased from left to right in Figure 41, i.e. as the amount of component II decreased and III increased. Again this was further tested by regression analysis of the mean M/F ratio for each blend against the amounts of each lactone present. The M/F ratio showed a significant negative relationship with the amount of II in the blend ($F=6.9$, $P<0.05$) (Fig.43a), and a positive (though not significant) relationship with the amount of component III ($F=4.9$, $P<0.1$) (Fig.43b). The level of IV, however, had no effect on the M/F ratio ($F=0.04$, $P>0.5$). The sexual differences in the responses therefore appeared to be affected by the ratio of components II:III in the blend (Fig.43c).

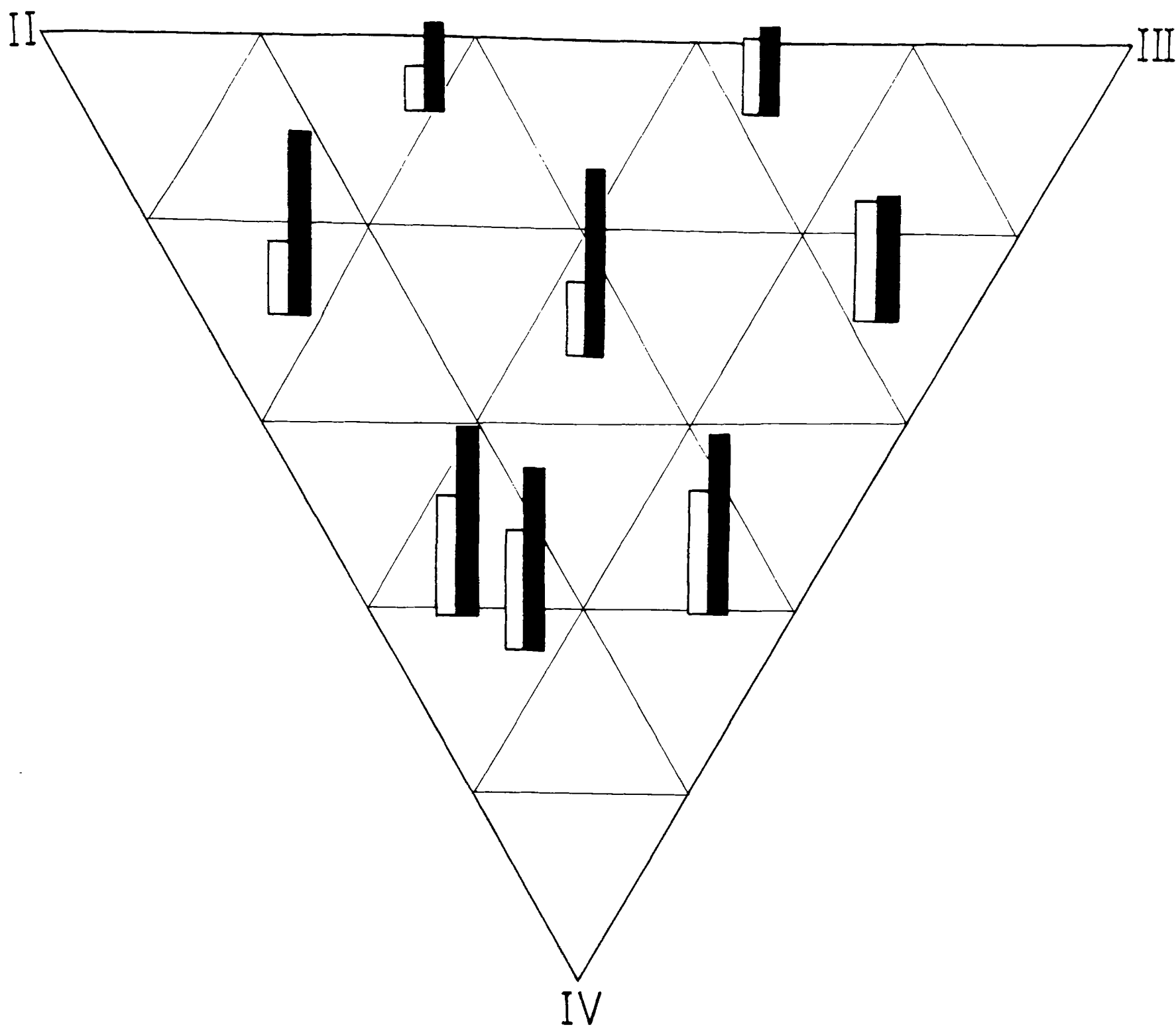


FIG.41 Effect of pheromone blend ratio on attraction of males (open blocks) and females (closed blocks). The position of the blocks corresponds to the blend ratio of the 3 components. Each corner represents 100% of the component indicated, falling to 0% along the opposite side of the triangle.

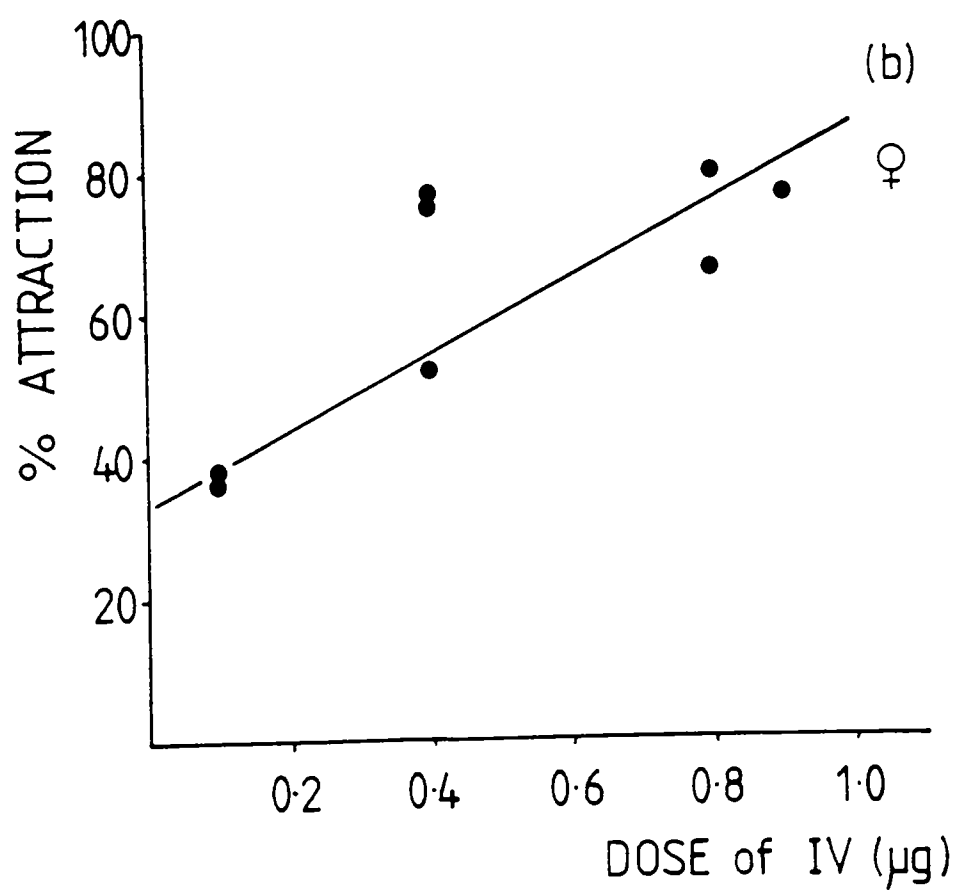
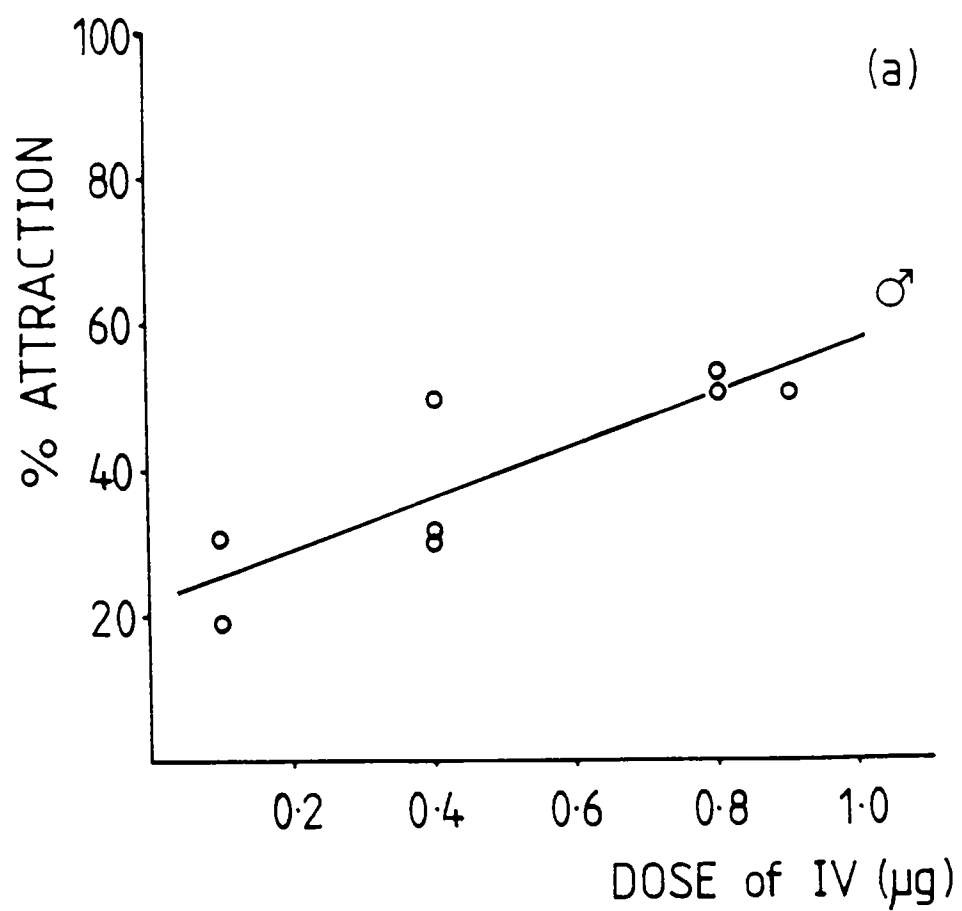


FIG.42 Effect of the amount of component IV in the pheromone blend on the attraction of (a) males and (b) females in the 2-choice pitfall bioassay.
 (a) Slope=0.35 %/ μg , significance of regression $F=12.7$, $P<0.001$.
 (b) Slope=0.45 %/ μg , $F=12.25$, $P<0.001$.

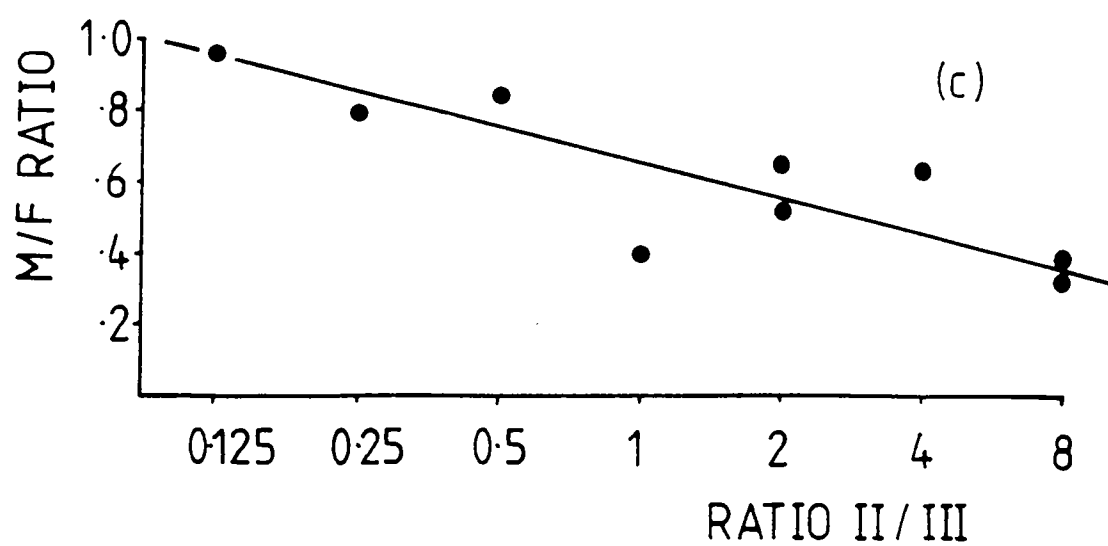
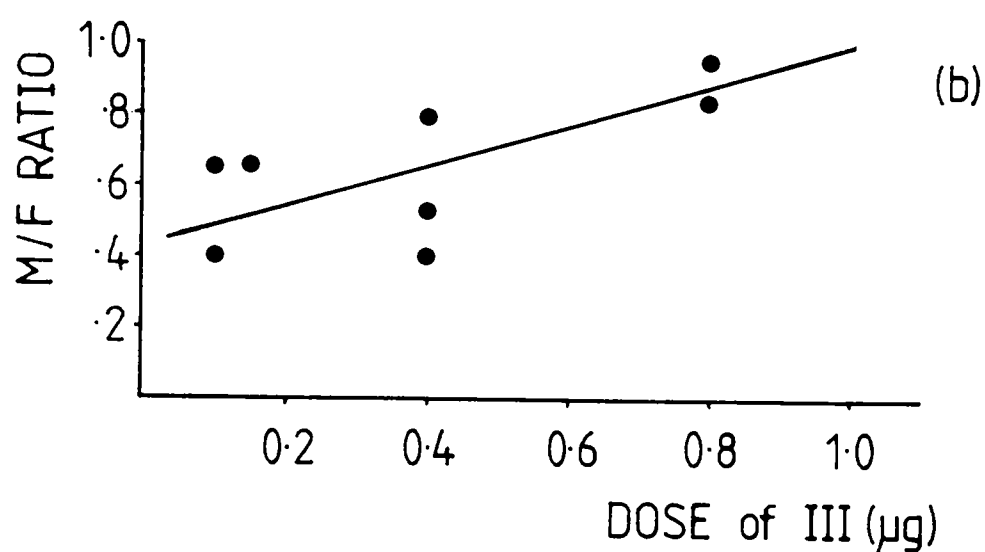
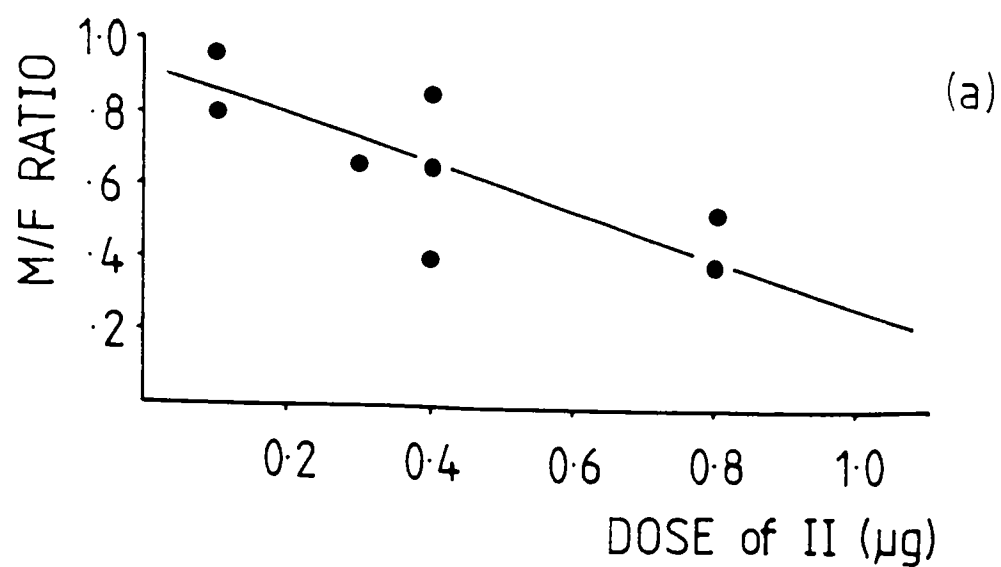


FIG.43. Effect of the amounts of components II and III in the pheromone blend on the male/female response ratio.
 (a) Effect of the amount of II.
 (b) Effect of the amount of III.
 (c) Effect of ratio of components II/III on the male/female response ratio.

DISCUSSION.

Although, as its name implies, the aggregation pheromone of O. surinamensis attracts both sexes, more females than males responded to the synthetic components and mixtures in all 4 experiments, suggesting that the male-produced blend does act in part as a sex pheromone. Pierce et al . (1984a) found a slightly greater response by females, but attributed no significance to this difference, and concluded that both sexes were equally attracted - in marked contrast to the results above. Millar and co-workers (1985a,b), however, found sexual differences in the responses of 2 related grain beetles Cryptolestes pusillus and C. turcicus . In the latter species females showed the greater response, whilst in the former, males responded at lower concentrations and had higher overall responses to the male-produced aggregation pheromone. This is inconsistent with the above hypothesis of a partial sex pheromone function in these grain beetles, and merits further investigation.

In O. surinamensis , the female response to the naturally produced ratio was greater than that of males, whether the insects were raised in single- or mixed-sex cultures. Culture type did have an effect, however, since fewer males from single-sex cultures were attracted than from mixed-sex cultures, yet vice versa for females. The effect was apparently stronger for males. Culture type could affect insects in several ways, including mated state (virgins in single-sex cultures, mated insects in mixed-sex cultures) and differences in pre-exposure to the pheromone, which would only be present in male and mixed-sex cultures. The results do show, however, the importance of standardising culturing methods and the mated state

of insects used for assaying aggregation pheromones as well as sex pheromones.

Although more females than males were attracted in all 4 experiments, the ratio of male to female responses varied with the components and mixtures tested. No mixtures, however, attracted more males than females. When components were omitted from the natural blend, the results suggested that the greater response of females was due to the presence of component II, since addition of this material to a mixture increased the numbers of females responding, but had no effect on males. The tests with different blend ratios also supported this, since the M/F ratio fell significantly as the amount of component II in the blend increased. The lack of differences between the sexes reported by Pierce et al. (1984a) could thus be explained by the lower level of this component in their test blend.

Since, at least in females, all 3 components are necessary to produce a strong degree of attraction, it appears that the mixture acts as a single entity, i.e. as a multicomponent pheromone, in agreement with the findings of Linn et al. (1984, 1986), and Baker and Carde (1979) working with moth sex pheromones. Amongst moths, it has been suggested that the blend ratio of the sex pheromone confers species-specificity on the signal (Baker et al., 1981; Linn and Roelofs, 1983), and this is important in closely related sympatric species that utilise the same pheromone components. In O. surinamensis blend ratio did affect the level of response of both males and females. Increases in the amount of component IV were found to have the greatest positive effect on the attractiveness of the

blend to both sexes, which was unexpected since when presented alone it produced no significant attraction. However, blend ratio was not that critical, since several markedly different ratios produced equally high levels of attraction, and furthermore, the naturally produced ratio did not produce the greatest mean attraction in either sex. This contrasts with the above studies of moths, where departures from the natural ratio often resulted in sharp decreases in the male response. It should be noted, however, that the present tests were conducted with relatively high doses and that blend ratio may be more critical at concentrations around the threshold level for detection.

The blend ratio of the aggregation pheromone does not, therefore, appear critical for species-specificity in these beetles. Specificity could be conferred by the chemical identity of the 3 components, since no other known species also uses this mixture. The actual blend ratio may be important in conveying extra information about the emitter, such as reproductive state, and this could explain the differential responses of males and females to changes in blend ratio. Such added complexity in aggregation pheromone communication systems might be expected since, not only are both sexes responding, but also, insects which utilise aggregation pheromones are generally long-lived as adults (Burkholder, 1982), and may need to alter the "meaning" (Slater, 1983) of the signal over time. In the boll weevil Anthonomus grandis, for example, the aggregation pheromone attracts both sexes, but at one time of the year acts as a sex pheromone and attracts mainly females (Mitchell et al., 1972). In this case, however, the change appears to be in the responsiveness of the insects rather than in the blend ratio.

Collection of volatiles (Chapter 5) suggested that the blend ratio produced by O. surinamensis cultures varied, but it was not possible to distinguish whether this was due to individual males altering the blend they released, or whether each male produced a fixed blend, and changes were caused by fluctuations in the numbers of males producing a given ratio. If males could alter the ratio they emit, theoretically they could affect the ratio of males to females likely to be attracted, perhaps drawing in females when sexually mature, or both sexes if habitat conditioning (e.g. by raising humidity) was required. Further research awaits the development of techniques to collect and analyse the pheromone blend produced by individual beetles.

CHAPTER 9.

AGGREGATION PHEROMONE: FACTORS AFFECTING PRODUCTION.

INTRODUCTION.

Production and release of beetle aggregation pheromones have been shown to be controlled by several physiological and environmental factors, including age, hormonal levels and circadian rhythms (Borden, 1974, 1985; Birch, 1984 and refs. therein). In many species studied, feeding has been found to be a prerequisite for pheromone release to occur. In the rusty grain beetle, Cryptolestes ferrugineus, for example, the availability of food increased the release rate of pheromone by up to 27-fold (Pierce et al., 1984b). A similar effect has been described in bark beetles (Scolytidae). Some species which attack healthy trees (e.g. Dendroctonus frontalis) seem able to release pheromone immediately on arrival at a suitable host (Renwick and Vite, 1969), but most species need to feed before attractive frass is produced (Pitman et al., 1965).

The timing of pheromone release, and the factors which affect this, should also reflect the function of the pheromone. If the pheromone acts to produce aggregations, as in bark beetles, pheromone production may well be inversely density dependent, since there is clearly an optimal density, above which food or space are likely to become limiting (Birch, 1984).

Behavioural experiments on the response of O. surinamensis to synthetic material (Chapter 8) suggested that the male-produced

aggregation pheromone in this species acted, in part, as a sex pheromone, since it preferentially attracted females. Previous work in both beetles and moths has shown that production and release of sex pheromones are often affected by the reproductive status of the emitter. In the drug-store beetle Stegobium paniceum , which utilises a female-produced sex pheromone, mated females were found to secrete less pheromone than virgins (Barratt, 1974). The pheromone content of the female moth Adoxophyes fasciata similarly dropped by around 120-fold following mating (Nagata et al. , 1972), whilst in Platynota stultana , the female "calling" behaviour associated with sex pheromone release did not occur in mated individuals (Carde and Webster, 1981).

The following experiment was conducted, therefore, to investigate the effects of density and reproductive status on pheromone production and release in individual saw-toothed grain beetles, to shed further light on the role of the pheromone produced by this species.

MATERIALS AND METHODS.

Measurement of pheromone release rates.

In most moths and certain beetles pheromone release involves a clearly defined "calling" behaviour which can be readily observed. In the common furniture beetle, Anobium punctatum, for example, females raise their abdomens and periodically extrude the pheromone-laden ovipositor (White and Birch, 1987). Aggregation pheromones in O. surinamensis and related species, however, appear to be produced in the alimentary canal, since they can be collected from both beetles and frass (Pierce et al., 1981), and so pheromone release cannot readily be observed. Consequently, previous studies of grain and bark beetles have measured release rates by the collection of volatiles from high density cultures (Chapter 5; Pierce et al., 1984a; Browne et al., 1974; Birch et al., 1980), but such large scale aerations cannot determine release rates from individual beetles. Techniques for collection of volatiles from individual grain beetles are under development, but are not available at present. Therefore, pheromone release by individuals was assayed using the response of conspecific beetles. This method does not directly measure pheromone release rates, neither can it detect differences in the blend ratio produced, but it does measure the net effect of all these variables on the behaviour of other beetles, which is the factor of principal biological significance.

Insect material.

Virgin insects were collected daily from cultures as they emerged as adults, sexed (Halstead, 1963) and either set up as odour sources,

or kept in single-sex cultures until used as test (i.e. responding) insects in the pitfall tests. Odour sources consisted of 4cm x 1cm plastic tubes containing insects with 1g of culture medium. Food was provided as it is essential for pheromone production in related beetles (Pierce et al ., 1984b). Source tubes were coated with Fluon at the neck and then covered by nylon mesh to prevent beetles either entering or leaving, but allowing volatiles to escape. Tubes contained either male, female or both sexes, at densities of either 1 or 10 insects per sex, per tube. Control tubes containing 1g of food alone were also set up at the same time, and all tubes were left for 14 days prior to testing. Responding insects were also used when 14 days old.

Test procedure.

The attraction of each sex to the source tubes was determined using the 2-choice pitfall assay (Chapter 4), with a source tube containing insects versus a control tube containing food alone in the 2 pitfalls (Fig.44). Each test was conducted with 10 responding insects in the arena, left for 1 hour in darkness. At the end of this time the numbers of responding beetles in each pitfall were counted, and the % attraction to the source tube containing the insects calculated.

Tests were run to determine the level of attraction of both male and female responding insects, to each of the 6 types of source tubes, with 10 replicates of each combination, in a fully randomised design. The results were tested by factorial analysis of variance to determine the effect of the source insects' sex and density on the attraction of males and females.

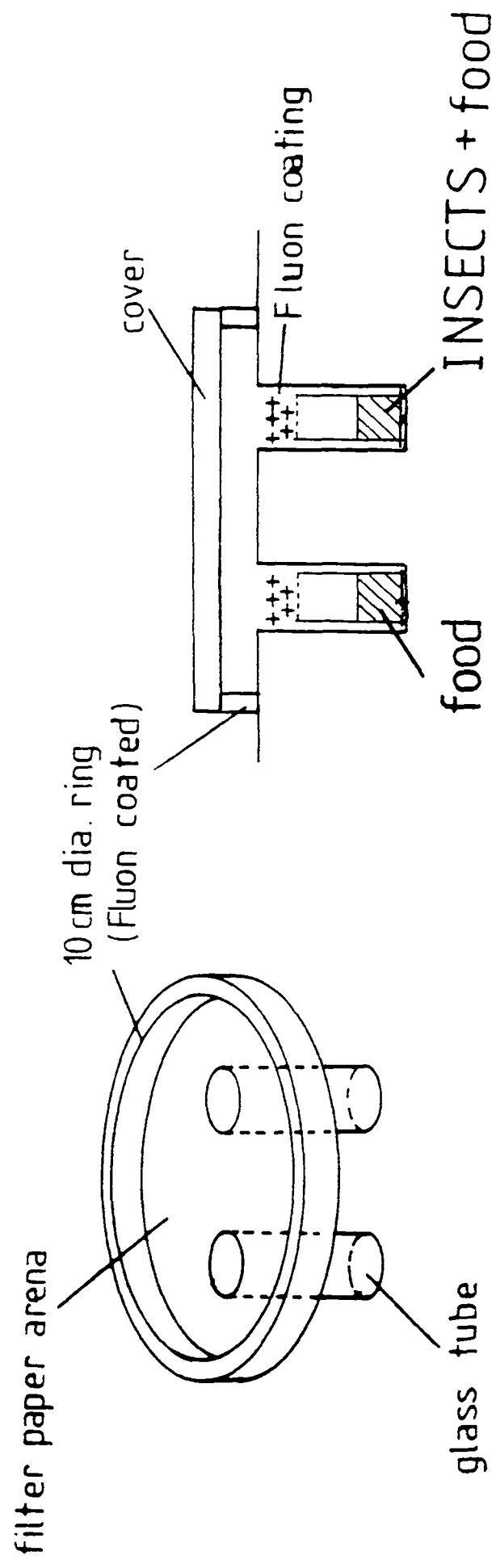


FIG.44 Two-choice pitfall bioassay apparatus used to investigate the factors affecting pheromone production.

RESULTS.

The sex of the source insects had a highly significant effect on the attraction of conspecific beetles ($F=21.5$, $P<0.001$) (Table 7.). When present alone, males attracted more individuals than either males with females, or females alone ($P<0.001$, Fisher's protected LSD test). There was also a significant interaction between the sex and density of the source insects ($F=10.0$, $P<0.001$). Single males were more attractive than tubes containing 10 males ($P<0.001$) whilst density had little effect on the attraction to female or mixed-sex tubes (Fig.45).

Table 7. Effect of Density and Sex of Source Insects on the Attraction of Conspecifics.

SOURCE	df	F
Density of Source insects (D)	1	1.0
Sex " " " (S)	2	21.5 ***
Sex of responding insects (R)	1	3.7
(D) x (S)	2	10.0 ***
(D) x (R)	1	1.3
(S) x (R)	2	1.4
(D) x (S) x (R)	2	0.8
Residual	108	
TOTAL	119	

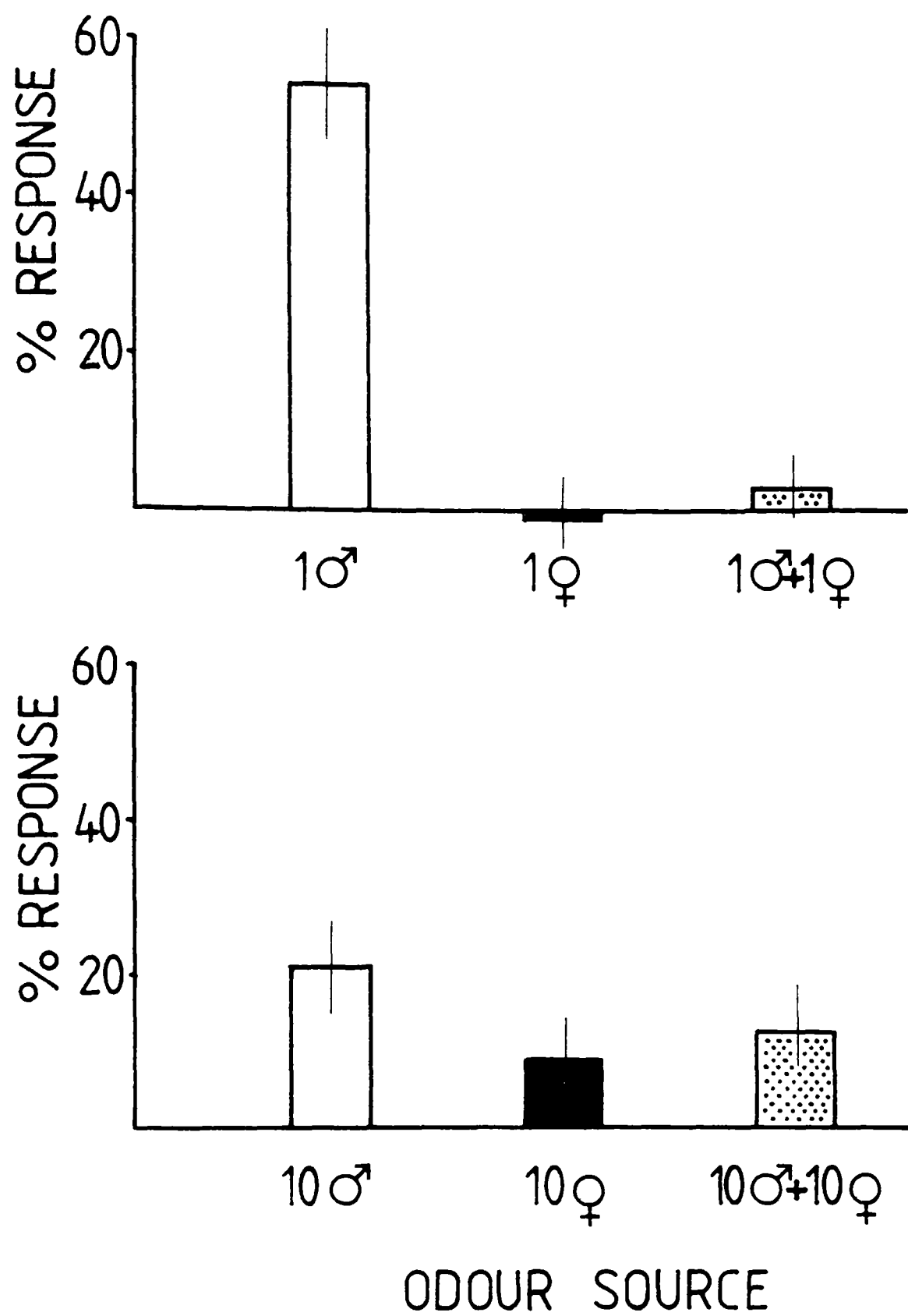


FIG.45 The effects of the sex and density of insects acting as an source on the attraction of conspecific beetles in 2-choice pitfall bioassay. Responses of males and females have been combined. (Means $\pm$ SE, 10 insects/test, $n=20$).

DISCUSSION.

The experiment confirmed earlier findings that in O. surinamensis, males produce an aggregation pheromone that attracts both sexes (Pierce et al., 1981, 1984a). However, this was the first direct demonstration of aggregation in Cucujid or Silvanid beetles, since previous work has involved the collection of volatiles from large insect cultures, solvent extraction and subsequent behavioural testing. The results also showed for the first time that attraction can occur to individual males, suggesting that single colonising beetles are able to elicit aggregations.

The results appear to contradict those reported above in Chapter 4, where insects on food in a pitfall tube were not found to be attractive to other insects. In that case, however, the insects and fresh food were placed in the pitfall tubes immediately before the tests were run. In the present case, the tubes were set up and left for 14 days prior to testing, which would allow pheromone production to occur.

Single males were considerably more attractive than the combined effect of 10 males, suggesting a very marked decrease in pheromone production per male with increasing density. (A possible alternative explanation is that the pheromone production of 10 males was so high that it inhibited attraction. However, in the behavioural tests reported above (Chapters 5, 8), this effect was not seen, even at the highest concentrations of synthetic pheromone mixture tested.) Using beetles of a similar age, Pierce and coworkers (1984a) found that pheromone production in large cultures fell by 50% if beetle density

was increased from 20 to 100 beetles /g. Such density-dependent production suggests that the pheromone acts to produce optimal densities in aggregations, and that once such levels are reached, further release is inhibited. The results also indicate that the laboratory methods widely used for collection of such pheromones (i.e. aeration of high density cultures) are inappropriate, and that aeration of fewer insects might produce greater amounts of material.

The second factor affecting the attraction to males was the presence or absence of females. If a female was present in the same source tube, the otherwise strong attraction to a single male was not significantly different from zero. This effect could have been caused in several ways. It^{is} unlikely that the increase in density, per se, was responsible, since the effect of adding one female to a producing male was equivalent to that of adding 9 males. A test with 2 males in a tube would be needed to conclusively rule out this possibility. Males in such mixed-sex tubes may partially or totally cease producing pheromone following mating, as shown in sex pheromone production by females in Stegobium paniceum (Barratt, 1974) and Adoxophyes fasciata (Nagata et al. ., 1972). However, as male saw-toothed grain beetles can and do mate repeatedly (pers. observation), the advantage of halting pheromone production once one female is attracted is not clear, unless this is only temporary and pheromone release restarts once the male is ready to mate again.

A second possible explanation for the observed low level of attraction to mixed-sex tubes could have been the release of additional behaviour modifying chemicals by the female which inhibited

the attraction to the male-produced pheromone. A similar inhibitory mechanism has been described previously in the Douglas fir beetle Dendroctonus pseudotsugae. Males responding to the female-produced pheromone arrive at the female's entrance hole and stridulate, causing her to release 3,2-methyl-cyclohexenone (3,2-MCH) (Rudinsky et al., 1973). Stridulation by females also stimulates males to release 3,2-MCH and 3,3-MCH, which both inhibit any further attraction to the female pheromone (Rudinsky et al., 1976; Libbey et al., 1976). If females did release an inhibitor, this would prevent attraction of further females and perhaps reduce competition for larval food sources, but would also prevent attraction of additional males and thus further possible matings.

A third possible mechanism could be the production of larval volatiles in the mixed-sex tubes (following mating, oviposition and subsequent hatching of the eggs) which could also act to inhibit the attraction to the pheromone. However, if either female or larval-produced inhibitors are produced, they would have been detected in the volatiles collected from cultures in Chapter 5. Consequently, the reduced attraction to single males in the presence of females was most likely due to a reduction in male pheromone production and release.

These results suggest, therefore, that pheromone production by male saw-toothed grain beetles was affected by both density, and also by the presence or absence of females, which in turn imply a sex pheromone as well as an aggregation-promoting function.

CHAPTER 10.

FACTORS AFFECTING THE ANTENNAL AND BEHAVIOURAL RESPONSE TO OLFACTORY STIMULI.

INTRODUCTION.

Behavioural responses to olfactory stimuli are known to vary with both environmental and physiological factors. The response of male Trichoplusia ni to its female sex pheromone, for example, has been shown to depend on the light intensity, time of day and the age of the male moths (Shorey and Gaston, 1964; 1965; Shorey et al. , 1968). Similarly, the response of male Trogoderma granarium to the female sex pheromone was affected by their sexual status, decreasing by about 50% after mating (Adeeson et al. , 1969). In O. surinamensis , the beetle studied here, Pierce et al. (1983) have reported that both age and population density affect the attraction of adults to beetle, frass and food volatiles.

Such changes in the behavioural response may be due to underlying changes in the sensitivity of peripheral olfactory receptors, or alternatively, variation in the central integration of the sensory input and generation of motor output. For some time, it has been generally accepted that the input from peripheral sensory receptors is relatively constant, and that the behaviour of an organism is controlled by processes in the central nervous system. However, several early attempts were made to identify changes in olfactory receptor sensitivity. Payne et al. (1970), for example, looked for variability in the EAG response of I. ni , but found that light

intensity, time of day and age did not affect the sensitivity of the olfactory receptors to the female sex pheromone. In the red-banded leaf roller Argyrotaenia velutinana , Roelofs and Comeau (1971) found very high variability in the responses of individual preparations, but only a very slight change in the EAG amplitude with age. Seabrook (1977) went as far as to state that the antennal response to sex pheromone is independent of age and environmental factors, an opinion repeated recently by Mayer and Mankin (1985). They claimed that the EAG should not be affected by physiological factors such as circadian rhythms (since such effects originate in the CNS, and so would not affect peripheral receptors) nor by age, once the olfactory receptor cells are fully developed at eclosion. However, they do admit that "some undescribed physiological phenomena do influence the EAG" which explains the high variability often found between individual preparations.

Evidence is now accumulating, however, for systematic variation in the sensitivity of peripheral olfactory receptors, and for correlation of such changes with observed changes in insect behaviour (Davis, 1984, 1986; Dickens, 1986). Variation in the antennal sensitivity to sex pheromone with age has been demonstrated at the EAG level in 3 species of Lepidoptera:- the codling moth Laspeyresia pomonella (Skirkevicius and Skirkeviciene, 1979) Choristoneura fumifera (Palaniswamy et al. , 1979) and Pseudaletia unipuncta (Seabrook et al. , 1979), and in worker bees (Masson and Arnold, 1984). Amongst the Coleoptera, the EAG response in the grape borer Xylotrechus pyrroderus to both the sex pheromone components and to a host-plant volatile were clearly shown to increase with age after

eclosion, although the effect of this on the insect's behaviour was not studied (Iwabuchi et al. , 1985). In the stable fly, Stomoxys calcitrans , Warnes and Finlayson (1986) found that the EAG response to carbon dioxide increased when flies were deprived of food, whilst Davis (1984) also found changes in sensitivity at the single olfactory receptor level in Aedes aegypti , which correlated with changes in the host-seeking behaviour of the female.

Similar variability has been found in studies of contact chemoreceptors. In Locusta migratoria , changes in the electrical resistance of gustatory sensilla, and variations in the proportion of sensilla responding to a stimulus have been reported, and such changes can explain the post-prandial rest seen in locust feeding behaviour (Bernays et al. , 1972; Bernays and Chapman, 1972). More recently, Schoonhoven et al. (1987) have shown that in larval Spodoptera littoralis , responses of chemoreceptors varied with the time of day, age and with the type of food on which they were reared, and that such changes could partly explain the variations observed in the feeding behaviour.

It appears, therefore, that in some cases, observed changes in the behavioural response to olfactory stimuli may result from alterations in the sensitivities of the peripheral olfactory receptors. The following experiments were conducted to determine which factors affect the behavioural response of O. surinamensis to food odour and to the aggregation pheromone, and to compare these with possible changes in the sensitivity of the olfactory receptors, as measured by the EAG response.

To validate the previous work of Pierce et al. (1983), the effects of age on the attraction to carob distillate and the aggregation pheromone was investigated using the multiple-insect pitfall bioassay. However, this method was not suitable for investigating many factors simultaneously, so a single-insect assay method was introduced, to test the effects of age, sex and density on the response to olfactory stimuli. Earlier work (Chapter 5) suggested that mated state also affected the response of insects to the pheromone, so this was also tested. For correlation with these behavioural results, variations in the EAG response to olfactory stimuli were determined between individuals of different ages, sexes and mated states.

MATERIALS AND METHODS.

Insect cultures.

Insects of known age were obtained by daily removing all newly emerged adults from several stock cultures, and setting them up in new culture jars. Every 2 weeks the culture medium was changed to prevent the development of another generation of adults. Cultures were kept at $25^{\circ} \pm 1^{\circ} \text{C}$, under a 16hr light: 8hr dark lighting regime (lights on 1800hrs, lights off 1000hrs).

Insects for the pitfall assays were kept in mixed sex cultures at a standard density of 200 individuals per 100g of food (2000 indivs/kg), and removed from the food and kept in clean empty culture jars for 24 h prior to testing.

Insects for the single-insect assays and the EAG recordings were sexed immediately after emergence (Halstead, 1963), and then cultured in 5 cm x 2.5 cm glass tubes at the standard density of 10 individuals per tube containing 5g of food (2000 indivs/kg). Each day 3 tubes were set up containing, respectively, virgin males, virgin females and mixed (and therefore presumed mated) males and females (5 males, 5 females per tube). This method appeared to give satisfactory results, since larvae developed in the tubes containing the mixed sexes, but not in those containing virgin beetles.

Insects for the single-insect assays on the effect of density on the behavioural response were cultured in similar tubes at 2 density levels:- 1 individual per tube (200 indivs/kg) and 20 individuals per tube (4000 indivs/kg) and were tested when 4 weeks old.

Behavioural assays.

i. Multiple-insect tests.

The effect of age on the behavioural response to food odour and to synthetic aggregation pheromone was tested using the pitfall assay described above (Chapter 4), with 20 insects of mixed sex per test, and 6 replicates for each age tested. Insects were tested over the age range 1 - 50 days following adult eclosion, this being the period over which the responses were shown to vary in previous work (Pierce et al., 1983).

The experiments were designed so that all ages were tested over the same period (2 days) to control for any variation in the test conditions or chemical stimuli with time. Tests were randomised so that all the beetles of the same age were not tested at the same time, which controlled for any effect of the time of day. Insects were used once only, and then discarded. All tests were conducted between 1400hrs and 1800hrs.

The source of food odour used was 5ul of carob distillate, with 5 ul of distilled water as the control in each test. The response to the aggregation pheromone was investigated using a mixture of the 3 synthetic lactones in the ratio 8:1:4 (56ng II:7ng III:28ng IV :- equivalent to c. 280 beetle hours) (see Chapter 5) in 10ul of pentane, with 10ul of pentane as the control.

ii. Single-insect tests.

An open arena assay was developed to investigate the responses of single insects to the food and pheromone odours. Each arena consisted of a perspex ring 10 cm in diameter, placed on an 11 cm diameter filter paper, resting on a sheet of glass. A 1 cm radius circle was

drawn lightly in pencil at the centre of the filter paper, and the arena was covered by a glass plate, giving an airtight seal.

Arena tests were conducted under red light at $25^{\circ} \pm 1^{\circ} \text{C}$ between 1000 and 1800 hrs. Single insects were removed from their culture tubes, placed in an arena and left for 10 mins to settle. At the end of this period the insect would either have come to rest at the edge of the arena, or would continue to walk slowly around the perimeter. The olfactory stimulus was then applied to a 1 cm square of filter paper, and the solvent allowed to evaporate. The square was placed in the circle at the centre of the arena, the arena covered and the clock started. Each insect was observed until it reached the source of the stimulus, i.e. crossed the central circle, and the time taken noted. The test was terminated if the insect had not responded in this way after 10 mins. The insect was then removed and discarded, the arena cover, ring and baseplate cleaned with hexane, and the filter paper replaced.

In this assay, the odour source was introduced into the arena, which was then sealed. Theoretically, a concentration gradient from the centre to the edge of the arena should have gradually developed by diffusion, and consequently, the odour concentration at the perimeter (the position of the insect at the start of the test) should have slowly risen. Under such circumstances, in the presence of the stimulating odour, the beetles moved away from the arena edge, and proceeded by a series of zig-zags to the odour source. Between each leg of the zig-zag, the beetle waved both antennae to either side of its path, suggesting orientation by transverse klinotaxis (Kennedy, 1986). Therefore, the time taken to reach the source in this assay was a combination of the time taken for the insect to detect the odour

(dependent on the sensitivity of the antenna and the source concentration) and the time taken to subsequently orientate up the odour gradient to the source.

The experiments were designed for a fully randomised 4 factor ANOVA. Tests were carried out using 3 different ages (2 days, 10 days and 28 days) of each sex, with both mated and virgin beetles and with 3 different concentrations of the olfactory stimulus. These ages were chosen as most likely to show changes in the behavioural response from the results of the multiple-insect tests (see below) and previous work (Pierce *et al.*, 1983). Beetles younger than 2 days after eclosion were not used because their cuticle had not hardened completely, and this may have affected their walking abilities. Six replicates were conducted for each of the 36 combinations of sex, age, mated state and concentration, giving a total of 216 tests in each experiment.

Separate experiments were carried out for the two odour sources using stimuli of CD/10, CD/100 and distilled water for carob distillate, and 140 bh, 1.4 bh equivalents and pentane for the synthetic pheromone mixture. Each experiment was conducted over 4 days, but complete randomisation of the testing order controlled for any effects of time on the response. To obtain beetles of each chosen age different culture tubes were used each day, which controlled for any effects of differences in culture conditions on the response.

A further experiment was conducted to investigate the effect of density on the response to the synthetic pheromone mixture. The insects used had either been cultured at 200 indivs/kg or at 4000 indivs/kg, and were tested against pentane and 140bh equivalents of

the synthetic pheromone mixture. Sexes were analysed separately, giving 8 combinations of density, sex and stimulus, with 18 replicates of each, again in a randomised design.

Antennal responses.

EAG responses were recorded using the standard procedure described above (Chapter 3). Each individual was presented with 7 concentrations of carob distillate followed by 8 concentrations of synthetic pheromone mixture, each in ascending order of concentration. In each case the blank response was subtracted from the observed EAG amplitude before analysis, but no normalisation of the results was used.

Ideally, the EAG response to all 3 pheromone components should have been determined separately to identify changes in sensitivity with any of the factors under study, but this was not possible due to the limited life of individual preparations. Therefore the response was measured to a 1:1:1 mixture of the 3 components, since this would show any differences in the antennal sensitivity to any one or more component. Use of a 8:1:4 blend would have detected changes in the response to the major component more readily than to the minor component.

Individuals were taken from the cultures set up above for the single-insect behavioural assays, using insects of both sexes, of 3 ages (2 days, 10 days and 28 days) with both mated and virgin beetles. Three replicates of each combination were tested, giving a total of 36 preparations.

Statistical analysis.

Results of both the single-insect assays and EAG experiments were analysed by factorial design analyses of variance (in the latter this was a 2 layer analysis to take account of the variance within and between individuals). In the single-insect assay results, however, the presence of several non-responders (i.e. response time 600 secs) in the results produced a right censored distribution, which questioned the use of a conventional parametric analysis of variance. The results were therefore also analysed by GLIM methods for survivorship data, which do not require a normal distribution of the data (Aitkin and Clayton, 1980). However, both methods gave similar results, and showed the same main effects and interactions. The results given below are for the analysis of variance method, since this allows direct comparisons between the behavioural and antennal responses.

RESULTS.

Behavioural assays.

i. Multiple-insect tests.

Insect age had a highly significant effect on the attraction to carob distillate odour in the 2-choice pitfall tests (Fig.46a). The youngest beetles tested (2 days) showed a significant preference for the food odour compared with the control ($P < 0.01$, Chi square test), and this response increased with adult age up to 16-20 days old, beyond which the response plateaued.

The attraction to the synthetic pheromone showed different variation with insect age (Fig.46b). Again, there was significant attraction ($P < 0.01$) in the youngest age tested (1 day), which increased initially with age until 4 days after adult emergence, but then gradually declined. There was evidence of variability in the response of older beetles, however, since a marked increase in response was shown at around 34 days.

ii. Single-insect tests.

The time taken for insects to reach the carob distillate odour source in the open arena assay was found to be significantly influenced by the age of the insect ($P < 0.001$), and by the concentration of the odour source ($P < 0.001$), but not by the insect's sex or mated state (Table 8, Fig.47a). Closer inspection of the results (Fig.47a) showed that there was some effect of age on the response to the control (i.e. water), but that the effect of age was greater on the response to the carob distillate odour. This suggested

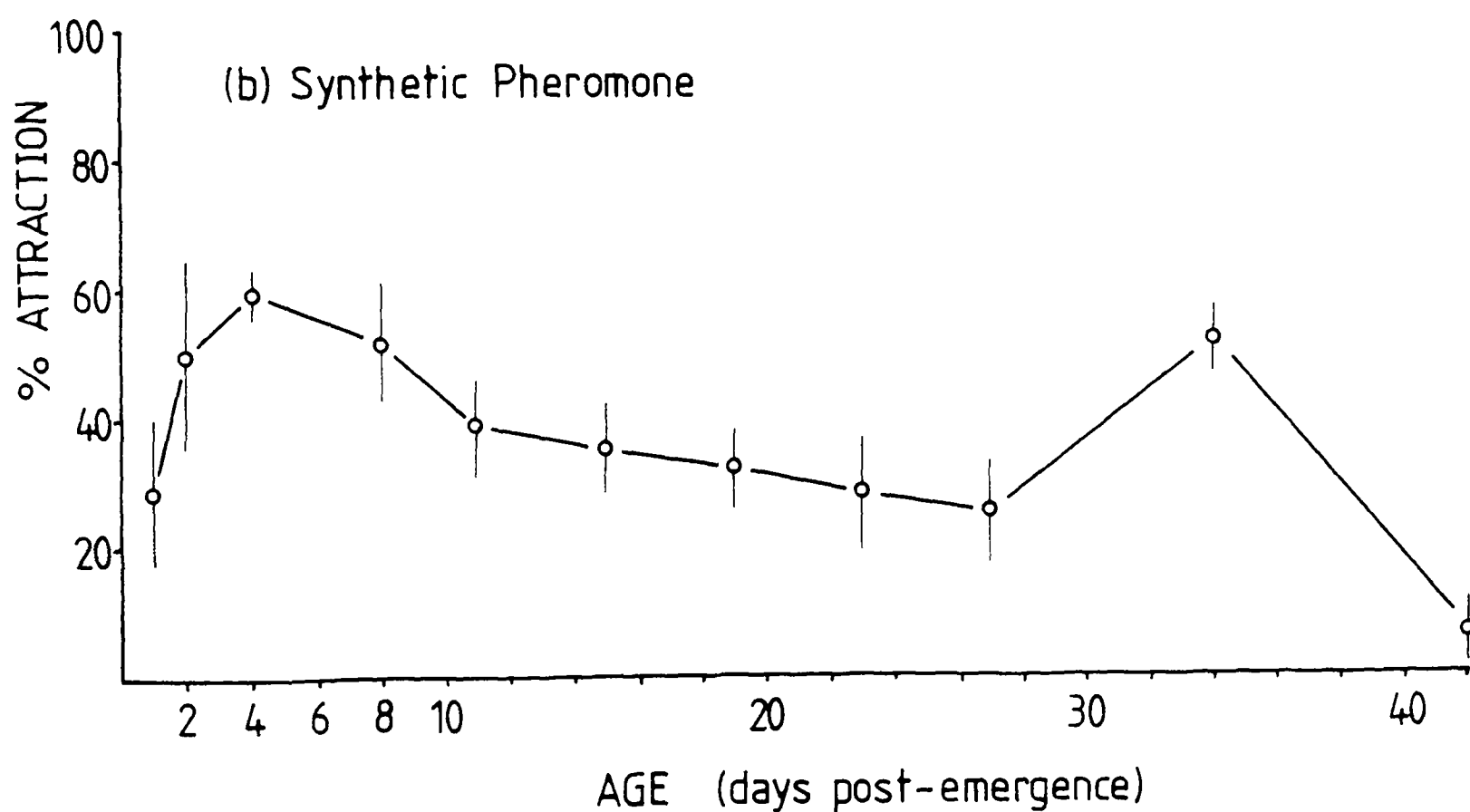
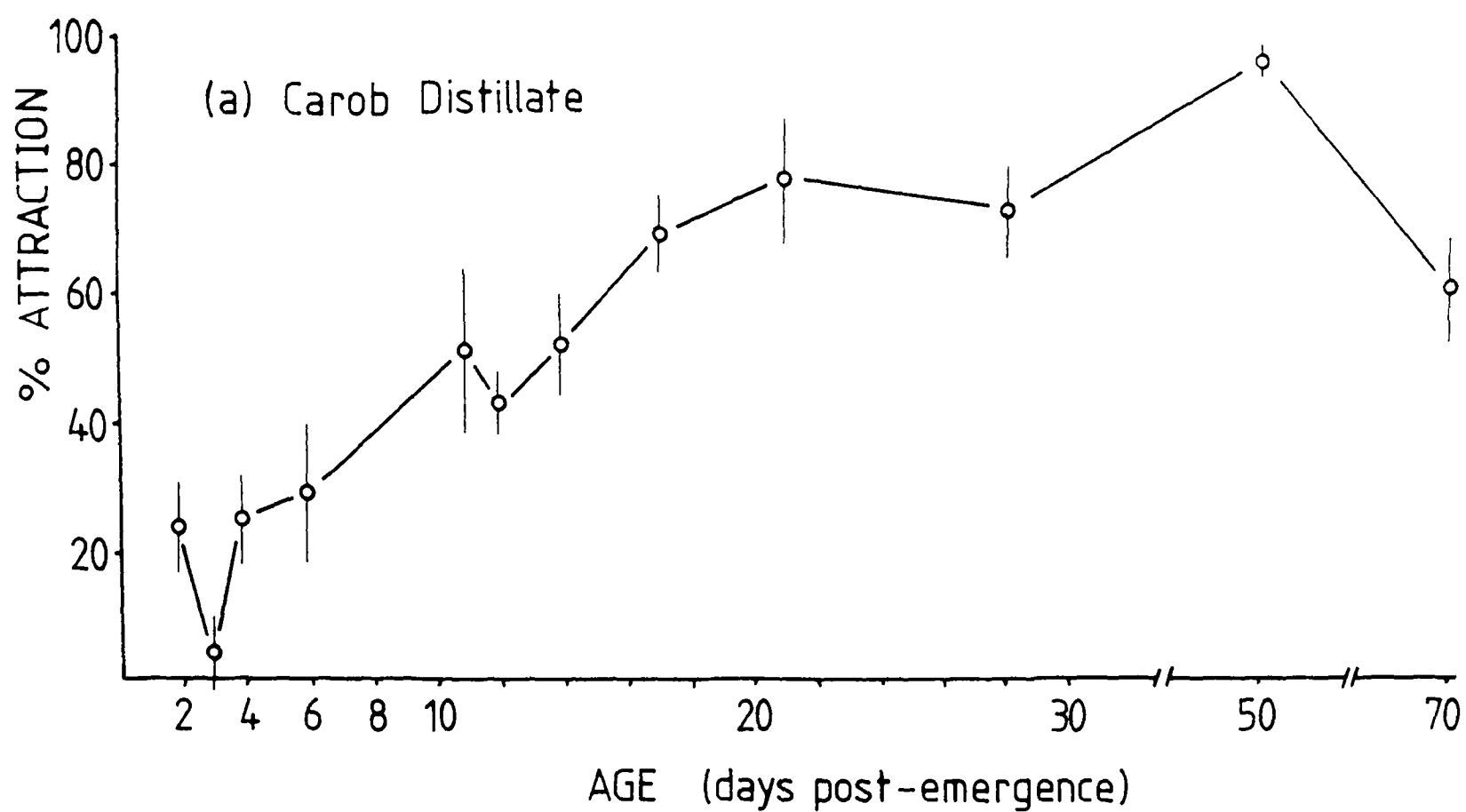


FIG.46 Effect of insect age on attraction to (a) Carob Distillate (5ul) and (b) Synthetic pheromone mixture (280bh) in the 2-choice pitfall bioassay. (Means $\pm$ SE, $n=6$ for each age tested).

that age affected the specific response to the carob odour rather than causing a more general effect on locomotory behaviour, which could theoretically also cause a decrease in the response time.

Although neither sex nor mated state were significant in the analysis as main effects, there was a highly significant interaction between these factors ($P<0.001$):- virgin males responded more rapidly to the food odour than mated males, whilst the reverse trend was shown by females (Table 9).

TABLE 8. Factors affecting the behavioural response to Carob distillate and synthetic pheromone.

		ODOUR		
		CD.		PHEROMONE
Source	df	F		F
SEX	1	0.6		7.2 **
AGE	2	7.8 ***		0.1
MATED STATE	1	0.1		0.0
CONCENTRATION	2	15.6 ***		23.1 ***
SEX x AGE	2	0.2		0.0
SEX x MATED STATE	1	12.9 ***		0.2
SEX x CONCENTRATION	2	2.4		0.7
AGE x MATED STATE	2	0.1		0.0
AGE x CONCENTRATION	4	1.2		0.8
MATED STATE x CONC	2	2.0		0.1
S x A x MS	2	0.9		2.8
S x A x C	4	0.9		0.8
S x MS x C	2	0.6		0.0
A x MS x C	4	1.7		0.3
Residual	184			
TOTAL	215			

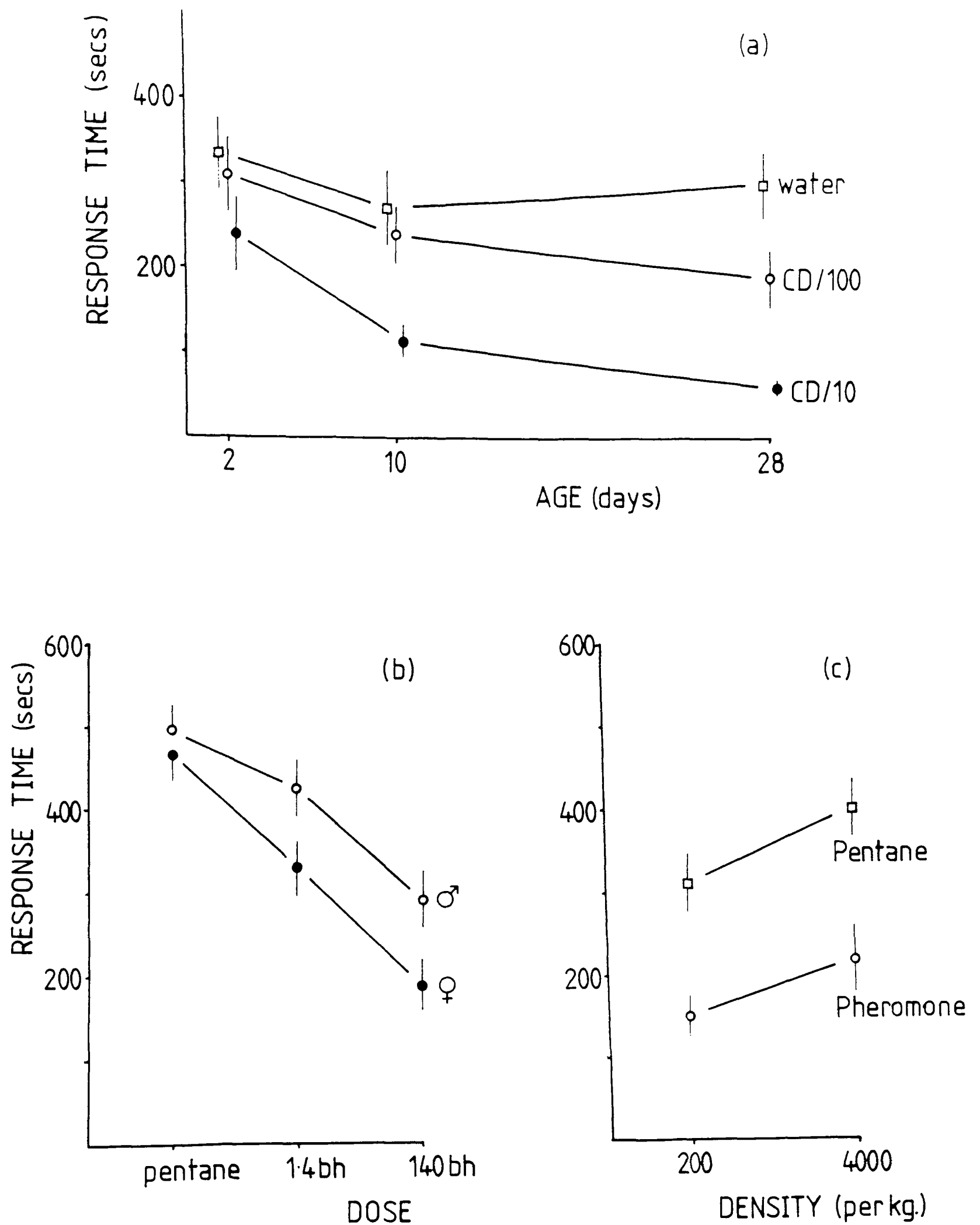


FIG.47 (a) Effect of insect age on the response of single insects to concentrations of Carob Distillate in open arena assay. (Means $\pm$ SE, $n=24$).
 (b) Effect of sex on the response of single insects to concentrations of synthetic pheromone mixture in open arena assay. (Means $\pm$ SE, $n=36$).
 (c) Effect of culture density on the response of individual insects to synthetic pheromone mixture (circles) and pentane control (squares) in open arena assay. (Mean $\pm$ SE, $n=36$)

TABLE 9. Effect of sex and mated state on the response to Carob Distillate.

	Virgin	Mated
Male	191.1 +/- 26.0	285.4 +/- 29.3
Female	259.5 +/- 29.0	181.1 +/- 21.1

(Figures are response times (secs.) +/- standard error)

In the analysis of the response of individuals to the synthetic pheromone source, sex of the responder was significant as a main effect ($P < 0.001$), with females responding more rapidly than males (Fig.47b). Again, detailed inspection of the results showed no difference between the sexes in the response to the control (pentane), but significant differences at both concentrations of pheromone.

Response time also varied with pheromone concentration ($P < 0.001$), but in contrast to the the results for carob distillate, there was no effect of age, nor any significant interactions.

In the separate experiment to determine the effect of insect density on the response to the synthetic pheromone, the response time was found to be significantly lower for beetles kept at low density ($F = 5.2$, $P < 0.05$). Further analysis of the effect, however, showed that the observed trend also applied to the response time to the pentane control (Fig.47c). This suggested that increases in culture density caused a decrease in the response to olfactory stimuli in general, rather than a decrease in the response to the aggregation pheromone in particular. The effect of density was therefore omitted from the investigation of antennal responses.

Antennal responses.

The EAG response to carob distillate was affected significantly by beetle age ($P<0.01$) and the concentration presented ($P<0.001$), but not by the beetle's sex or mated state (Table 10). There were significant interactions between age and concentration ($P<0.001$) (Fig.48a) and between sex, mated state and concentration ($P<0.01$) (Figs.49a,b).

TABLE 10. Factors affecting the EAG response to Carob Distillate and Synthetic Pheromone.

Source	Carob.		Pheromone.	
	df.	F.	df.	F.
SEX	1	1.0	1	0.4
AGE	2	6.2 **	2	2.1
MATED STATE	1	0.4	1	0.7
SEX x AGE	2	0.3	2	0.0
SEX x MATED STATE	1	1.9	1	2.8
AGE x MATED STATE	2	0.0	2	0.0
SEX x AGE x M.STATE	2	0.3	2	0.9
Residual 1	24		24	
Individuals	35		35	
CONCENTRATION	6	176.9 ***	7	123.2 ***
SEX x CONC	6	1.5	7	0.4
AGE x CONC	12	4.7 ***	14	1.1
MATED STATE x CONC	6	0.3	7	0.7
SEX x AGE x CONC	12	0.3	14	0.6
SEX x M.STATE x CONC	6	4.0 **	7	2.0
AGE x M.STATE x CONC	12	0.5	14	0.3
Residual	156		182	
TOTAL	251		287	

In contrast, the EAG response to the mixture of the synthetic pheromone components was not affected by sex, age or mated state, nor were there any significant interactions (Table 10). The only factor affecting the EAG response was the concentration presented ($P<0.001$) (Fig.48b).

Analysis of the mean EAG solvent (pentane) blank response from

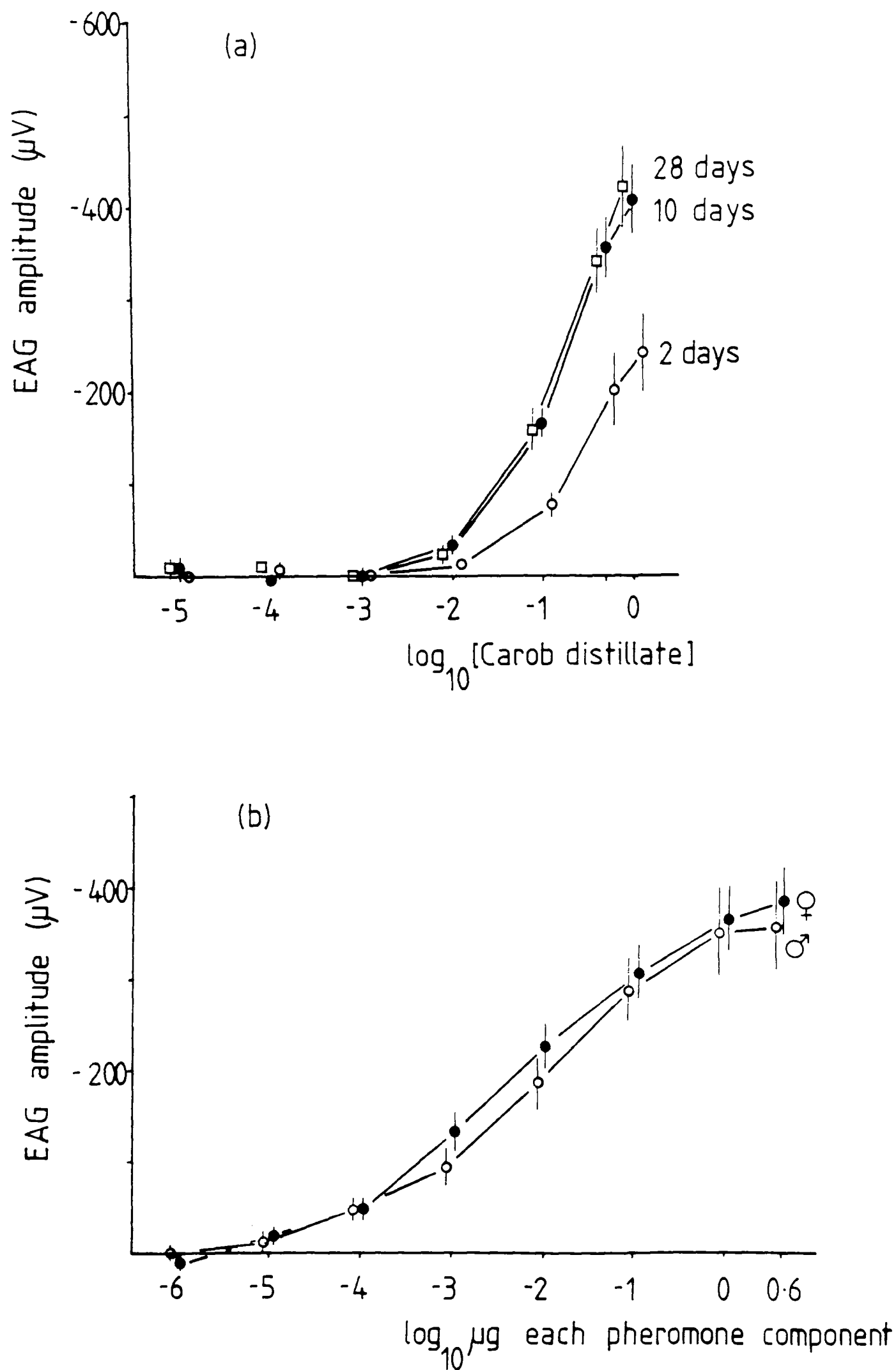


FIG.48 (a) Effect of age on the EAG response to Carob Distillate. (Means $\pm$ SE, $n=12$)
 (b) Effect of sex on EAG response to synthetic pheromone mixture. (Means $\pm$ SE, $n=12$).

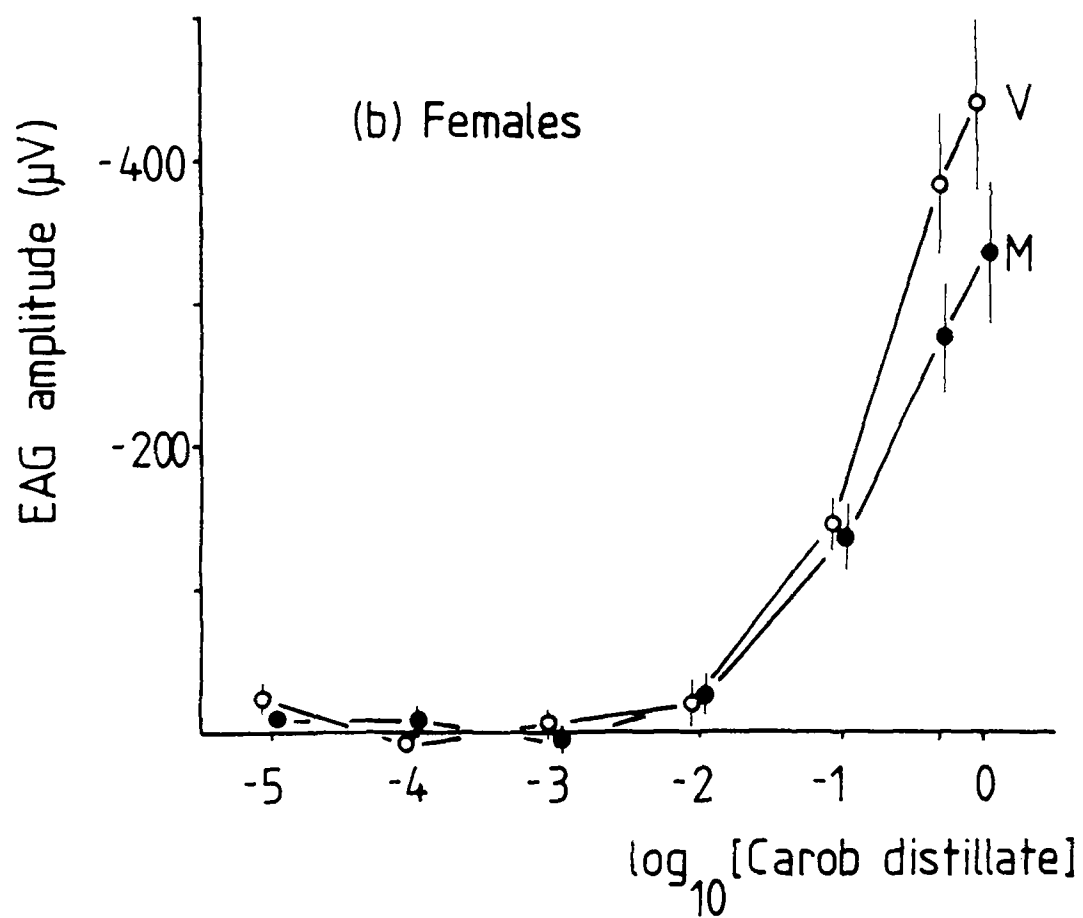
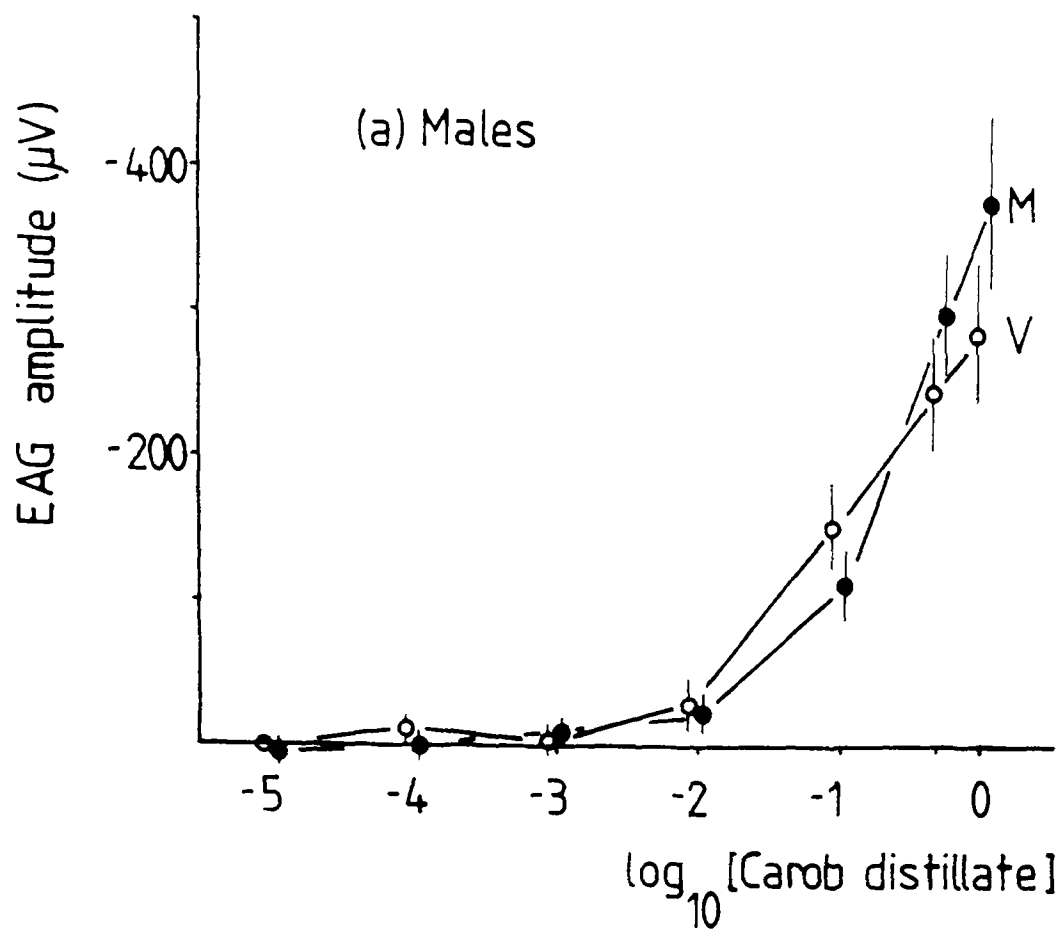


FIG.49 Effect of mated state on the EAG response to Carob Distillate in (a) males and (b) females. M=mated, V=virgin. (Means $\pm$ SE, $n=9$).

each preparation did show an effect of age, but not with sex or mated state of the beetle (Table 11). The blank response rose significantly from 2 days (response = 35.8 ± 10.6 uV) to 10 days (240.3 ± 24.4 uV), but there was no difference between 10 and 28 day old beetles (237.0 ± 18.5 uV).

TABLE 11. Factors affecting the EAG response to solvent blank.

Source	d.f.	F.
SEX	1	1.1
AGE	2	37.9 ***
MATED STATE	1	2.0
SEX x AGE	2	0.1
SEX x MATED STATE	1	2.5
AGE x MATED STATE	2	0.5
SEX x AGE x MATED STATE	2	0.4

Note that the EAG responses to the carob distillate and pheromone were corrected for the blank response before analysis, so the effect of age on the response to the carob odour was independent of the effect of age on the response to the blank cartridge.

DISCUSSION.

Both the pitfall and the open arena assays showed that the attraction of O. surinamensis to carob odour increased with age. Since the two tests measured different aspects of the response (numbers of insects responding and rate of response in individual insects, respectively) yet both showed the same trend, suggests a strong influence of age, which may be explained by reference to the reproductive activity of these beetles. Adults are capable of mating within 2 days of eclosion (Pierce et al. , 1981), and the females commence oviposition within 3-8 days (Howe, 1956). Maximal egg laying rates are normally attained after one week and maintained for a month (Howe, 1956), although under favourable conditions, oviposition may continue for over 100 days (Arbogast, 1976). Reproductive activity therefore starts soon after eclosion and peaks, at least for females, within the first month of adult life.

Long-lived beetles, such as O. surinamensis , need to feed as adults in order to reproduce (Burkholder, 1982). Food may also be necessary for pheromone production in males, since in the related beetle Cryptolestes ferrugineus , pheromone production is greatly enhanced when food is available (Pierce et al. , 1984b). The increase in the attraction to the food odour may therefore reflect an increased food requirement due to the rise in reproductive effort over the first month of adult life.

Similar factors may also be responsible for the observed interaction of sex and mated state on the response rate to carob distillate in the open arena assay (Table 9). Greater effort expended in egg production could explain why mated females responded more

rapidly to the food odour than do virgin females. Furthermore, if the male-produced pheromone has a mate-attracting function (Chapter 5), virgin males may have higher rates of pheromone production than recently mated males, as suggested in Chapter 9.

The above results differ, however, from the previous study (Pierce et al., 1983), which used brewer's yeast volatiles as a food odour. Using beetles cultured at a similar density (1000 indivs/kg), they found an initial negative response to food volatiles, followed by a refractory period where there was no significant response in either direction. They found a similar result with frass and beetle-produced volatiles, with a positive response on the first day after eclosion, followed by a period of negative or no response. Further positive responses only developed after 30 days in beetles kept at 1000 indivs/kg, and took even longer at higher densities. Thus, during the period of maximum reproductive activity, beetles were unresponsive to either the aggregation pheromone or food odour, and this was interpreted as a mechanism to promote dispersal at this stage (Pierce et al., 1983).

Several factors could explain the disparity in the results, including differences in the bioassay method and the behaviours assayed (Chapter 4), the use of different food volatiles or a difference between the populations of insects used, but it is difficult to understand why female beetles in the study of Pierce et al. were not attracted to the male-produced pheromone when they were reproductively mature.

The results presented above (Fig.46b, Table 8) for the behavioural response to the synthetic pheromone show no evidence of a

refractory period, however. The multiple-insect pitfall test showed an initial increase in attraction, which would correlate with the onset of sexual maturity, but there was a positive response at all but the oldest age tested. In the single insect assay, insect age did not affect the response rate, but again there was a positive response at all 3 ages tested. The single insect assay did, however, show a significant sexual difference in the response, which supports the greater response of females previously shown in the pitfall tests (Chapter 8). Therefore, although their responses differ, both sexes of O. surinamensis respond to the male-produced pheromone almost immediately after emergence, and continue to respond for at least the period of maximum reproductive activity - a strategy found in several other stored-product coleopterans, including Tribolium castaneum (Faustini et al. , 1981), Sitophilus granarius (Faustini et al. , 1982), Rhizopertha dominica (Khorramshahi and Burkholder, 1981) and Oryzaephilus mercator (Pierce et al. , 1984b).

Contrary to previous claims of the independence of EAG responses from environmental and physiological influences (Seabrook, 1977; Mayer and Mankin, 1986), the antennal responses were shown to be affected by several factors. Increase in age caused a significant increase in the EAG response to both carob distillate, and to the blank cartridge, but not to the pheromone. This increase occurred between 2 and 10 days following emergence, with no further increase between 10 and 28 days. An increase in the EAG response over the first few days of adult life has been reported in other studies (Palinismamy et al. , 1979; Seabrook et al. , 1979; Masson and Arnold, 1984; Dickens, 1986). As the EAG magnitude has been proposed as a relative measure of the

number of acceptor sites for any given odour (Dickens and Payne, 1977; Payne, 1975), such a change has been equated with an increase in the number of sites (Seabrook *et al.*, 1979). However, it is not possible to distinguish between an increase in the number of acceptor sites or an increase in the response produced at each site, or a combination of both.

The effect of age on the blank response is also interesting but ambiguous, due to insufficient knowledge of the origin of the observed EAG. It has been suggested above (Chapter 3) that the blank response represents combined mechanoreceptor and general olfactory receptor responses. Age may affect either one or both of these components.

The EAG response to the synthetic pheromone did not, however, alter with age, suggesting that these receptors are fully functional by day 2. Thus there is a differential change in the sensitivity of the olfactory sensilla, depending on their specificities. In functional terms, this means that the antenna is maximally sensitive to the aggregation pheromone by 2 days after eclosion, when mating can occur, whilst the sensitivity to food odour continues to develop.

Of the other factors affecting the EAG response, only odour concentration affected the response to both food odour and pheromone. Beetle sex had no effect on the response to either stimulus, which is consistent with the results presented above (Chapters 3 and 6).

Comparison of the factors affecting the antennal and behavioural responses to food odour and pheromone suggests that part of the behavioural variation observed may be explained in terms of changes in the sensitivity of peripheral olfactory receptors. The increase in the behavioural response to carob odour, but not to the synthetic

pheromone, with age correlates with the respective EAG recordings. However, the correlation is not perfect since the behavioural response to carob continued to increase from 10 to 28 days in both assay methods, but no further increase occurred in the EAG amplitude. Similarly, the interaction between sex and mated state on the behavioural response to carob distillate correlated with the interaction between sex, mated state and concentration on the EAG response. However, the effects were in opposite directions i.e. beetles responding more rapidly in the arena assay had lower and not higher EAG responses - a result that is hard to explain. Furthermore, the sexual differences in the behavioural response did not appear to be due to any difference in antennal sensitivity.

The main factor shown to influence antennal sensitivity and behaviour, therefore, was age, which appeared to reflect the development of the sensitivity of the receptors for food odour. In a similar way, Davis (1984b) showed that the onset of host-seeking behaviour in the female mosquito Aedes aegypti correlated with the development of the response of individual lactic acid receptors on the antenna. However, he also demonstrated that short term reductions in host-seeking behaviour following a blood meal also correlated with a depression in the sensitivity of these receptors, which was in turn due to a haemolymph-borne factor (Davis, 1984a). Other examples of short term changes in receptor sensitivity due to haemolymph-borne factors have been reported in the spruce budworm moth (Palaniswamy et al., 1979), and locusts (Bernays, 1980; Bernays and Chapman, 1972; Bernays et al., 1972). In O. surinamensis, food deprivation causes an increased response to food odour in the open arena behavioural

bioassay (White, unpublished data). It will be interesting to see whether this correlates with an increase in the antennal sensitivity to food odour, and if so, whether this is due to factors carried in the haemolymph.

CHAPTER 11.

GENERAL DISCUSSION.

A principal aim of this study was to obtain an integrated picture of insect olfaction by investigating the responses of a single insect species at the structural, electrophysiological and behavioural levels. Since each area has been discussed separately in the relevant section, this final chapter concentrates on the correlation between such levels.

There was generally good agreement between the distribution of antennal sensilla and the antennal (EAG) responses recorded. The amplitude of the EAG response, for example, could be related to the numbers of sensilla on the antenna when these were experimentally varied (Chapter 3). No sexual dimorphism in either numbers or distribution of olfactory sensilla was found, and, similarly, no sexual differences were found in the EAG responses to any food odour, aeration extract or pheromone component or mixture, despite numerous tests. In his review of chemoreceptor numbers, Chapman (1982) proposed that a lack of any differences in sensilla numbers between the sexes suggested that either olfaction is not important in the location of mates or courtship, or that both sexes respond to the same chemical stimuli. Clearly, equal numbers of olfactory sensilla is not proof of a lack of sexual differences in antennal responsiveness, since the specificities of receptors cannot be determined in a purely structural study. In the Anobiid beetle Anobium punctatum, for example, preliminary SEM studies failed to detect any antennal sexual

dimorphism (White, unpublished data), yet both EAG and behavioural studies showed that males were markedly more responsive to the female sex pheromone (White and Birch, 1987). In O. surinamensis, however, lack of difference in both structure and EAG response does suggest that both sexes respond to the same olfactory stimuli, but as shown above, the level of behavioural response varied.

The second interesting result of the structural study was the paucity of antennal olfactory sensilla, compared to those for mechanoreception, suggesting a low level of sensitivity to odours. As suggested above, the low number of receptors may simply reflect the small size of these insects. However, large numbers of sensilla, and hence high sensitivity, are usually associated with strong fliers that are capable of reaching distant odour sources (Chapman, 1982), since there would be little benefit in detecting sources that could not be reached. Although the strain of O. surinamensis used in this study possessed fully formed hind wings, it could not be induced to fly in the laboratory and so could fall into this category. However, the closely related species, O. mercator flies to light traps (Halstead, 1980), yet appears to possess a similar array of antennal sensilla (pers. observation). Antennal (EAG) recordings also appeared to contradict the notion of low sensitivity to odours, since the threshold for a response to the pheromone components compared favourably with results from insects with considerably more olfactory sensilla. Unfortunately, it was not possible to compare the antennal sensitivity to food odour with that to pheromone, since the concentrations of the active components in carob distillate were not determined. However, the larger maximum EAG response to this stimulus

suggested that there were relatively more acceptors for food odours than for pheromone components (Dickens and Payne, 1977), which could lead to greater antennal sensitivity to food odour than to pheromone, a phenomenon not commonly found in insects.

Behavioural sensitivity to odours depends not only on the numbers and sensitivity of olfactory receptors, but also on the levels of convergence and central integration of olfactory pathways in the central nervous system (CNS). However, comparisons between the sensitivity of antennal and behavioural responses are fraught with difficulty due to differences in the odour presentation methods. In the former, for example, antennae were fixed in position during recordings, whilst in the latter, freely walking beetles continuously waved their antennae, thus sampling a greater volume of air, and probably increasing the level of sensitivity. It was possible, however, to show a behavioural response to doses of synthetic pheromone mixture as low as c. 0.3ng (1.4bh) in the open arena assay (Chapter 10) and to the volatiles released by single male insects (Chapter 9).

Other comparisons between antennal (EAG) and behavioural responses were easier to make. For example, with food odours, there was close agreement between the relative EAG response and the level of attraction produced. Also, the lack of sexual difference in the antennal responses was mirrored by the same result for the behavioural responses. This correlation also applied to the factors affecting the EAG and behavioural responses, since insect age was found to affect both the EAG and behavioural responses to carob distillate in a

similar manner. Such good agreement between the behavioural and EAG responses does not always occur, since the EAG provides a quantitative, rather than a qualitative measure of the antennal response. It measures the sum of all the receptor potentials produced, but does not distinguish which receptors are stimulated. Boeckh (1986) cautioned against the uncritical use of the EAG as a standard test of the sensory effectivity of compounds on an antenna. Important components might act on a few receptors only, and so not cause a large EAG response. However, in defence of the EAG technique, selection should act to produce high sensitivity to components that are important long-range attractants, which should lead to large numbers of such receptors, and hence a potentially large EAG response.

In contrast~~to~~ food odours, however, little agreement could be found between aspects of the EAG and behavioural responses to the aggregation pheromone (although the mixture of components caused both attraction and EAG responses). The marked sexual difference in the response to synthetic pheromone, which was found in 4 separate experiments involving 2 different assay methods, was not accompanied by any difference in the EAG responses recorded. Behavioural investigations into the response to individual components and blends (Chapter 8) suggested that the relative response of the sexes depended on the ratio of 2 components (II and III) in the blend, but again, neither component could be shown to produce differential EAG responses in males and females. This suggests that sexual differences in central integration of olfactory inputs from pheromone receptors were responsible.

Central integration is clearly implicated in producing the

behavioural response to the pheromone since the insect responded to the whole blend, rather than to individual components. Component IV, for example, had little effect on attraction when presented alone, yet had a marked effect when presented simultaneously with either II (females) or III (both sexes) (see Chapter 8). This integration of inputs did not appear to occur at the peripheral level as has been suggested in some species (O'Connell, 1986) as separate receptors were found for each component.

The apparent difference in the level of central integration of pheromone, as opposed to food odour inputs, suggests basically different central pathways for such stimuli, and previous structural and electrophysiological work on olfactory pathways support this view. The axons of antennal olfactory receptor cells form part of the antennal nerve and terminate in the antennal lobe, in tangles of axonal and dendritic connections termed "glomeruli" (Mustaparta, 1984; Hildebrand and Montague, 1986). Two types of glomerulus have been distinguished:- a distinctly larger "macroglomerulus", which receives the axons from pheromone sensitive antennal receptors, and numerous "ordinary" glomeruli which receive axons from other olfactory receptors (Camazine and Hildebrand, 1979; Boeckh and Boeckh, 1979; Mustaparta, 1984). There is thus separation of the olfactory inputs from food odours and pheromones at the first stage of central integration. Such studies of olfactory pathways and antennal lobe structure have involved insects such as moths (Manduca sexta , Hildebrand and Montague, 1986; Antheraea sp., Boeckh and Boeckh, 1979) and cockroaches (Burrows et al ., 1982) which utilise female sex pheromones, and have shown sexual dimorphism in the antennal lobe,

since only males possess the macroglomerulus structure. No work appears to have been done on insects which utilise aggregation pheromones. It would be interesting to determine whether initial integration of pheromone inputs in O. surinamensis also occurs in a distinct macroglomerulus, and whether the observed differences in the behaviour are related to sexual dimorphism in the antennal lobe, rather than in the antenna.

Finally, the range of studies presented above also allow for a discussion of the evolutionary and functional significance of both food odours and pheromones to this beetle. Of prime importance to this is a consideration of the distribution of odour sources in field situations, and the distance over which attraction to such sources can occur. The latter depends on the sensitivity of the behavioural response to odours which has been discussed above. Both behavioural assays utilised in this work involved small arenas producing still air conditions, since this was considered to approximate to the situation within bulks of grain. Insects were able to orient to the odour source using the odour gradient produced in such arenas i.e. by chemotaxis. Observation of the "wig-wagging" behaviour of beetles in the arena suggested that insects were indeed utilising the odour gradient, by the mechanism of transverse klinotaxis (Kennedy, 1986). The distribution of olfactory sensilla i.e. only on the terminal club segments, is also ideal for the detection of concentration differences in an odour gradient. However, even in still air conditions, gradients only exist in the immediate vicinity of an odour source, and so this mechanism cannot explain attraction of individuals over large distances within grain stores, and even less the location of the grain

stores in the first instance. Distant attraction would require the use of alternative mechanisms, such as odour conditioned anemotaxis, although there is no evidence that this occurs in O. surinamensis .

Large modern commercial stores probably represent strong and continuous sources of odour, but insects are most likely distributed between stores in grain shipments. However, prior to the existence of such stores, many stored-product pests probably infested small domestic stores, or animal nests, both of which would represent scarce and isolated habitats. The need to locate such habitats could explain the relatively high number of antennal receptors for food odour found in this study. Aggregation pheromones have also been suggested as indicators of a potentially suitable food source (Shorey, 1973; Borden, 1977), since pheromone production appears to be dependent on the presence of food (Pierce et al ., 1984b). In such cases, insects might be expected to respond also to the pheromones of other species which infest similar foodstuffs. Whilst no behavioural experiments were attempted, the EAG responses to such materials (Chapter 7) did not support this. Food odours and pheromones could interact, however, to augment the response. Many species of bark beetles, for example, incorporate host tree odours as part of their "pheromone" blend (Birch, 1984). To date, no studies of the interaction of food odours and pheromones have been conducted on stored-product insects (Borden, 1985), so this could be a fruitful area for future research.

Within a grain store, aggregations are likely to be caused by the beetle-produced pheromone, although other factors such as high humidity are also known to attract beetles (Stubbs and Griffin, 1984). As the pheromone attracts both sexes, large mixed-sex aggregations

could result, which might function to cause local heating and an increase in humidity which would benefit larval survival and rate of development. The apparent decrease in pheromone production at high densities (Chapter 9) would set an upper limit to such aggregations. The experiments conducted on pheromone production and response, however, suggest considerable complexity in the mode of action of this aggregation pheromone. Several findings support the idea that the pheromone may act in part as a sex pheromone. Firstly, it is only produced by males, and all experiments with synthetic pheromone demonstrated that females were preferentially attracted. Interestingly, the only case where no sexual difference was found in the behavioural response was in the test using actual insects as the odour sources, where the difference fell just below the level of significance. However, in this case the materials released by the males, and the blend ratio, were not known. Secondly, pheromone production by males appeared to be affected by the presence of a female, and/or the reproductive state of the male. The mechanism responsible for this effect and the way in which this, and density, affect pheromone production in natural aggregations merit further research.

The aeration experiments suggested that the blend ratio produced by large cultures of insects may not be fixed. This could have been caused by differences between the blends produced by individual males, or by individual males changing the blends that they produce. Furthermore, behavioural tests showed that changes in the blend ratio altered the ratio of females:males that were attracted to the pheromone. Therefore, either different males will attract a different ratio of females:males, or individual males may be able to alter the

relative attractiveness of their pheromone blend to males or females over time. Thus the pheromone could act primarily as either an aggregation pheromone, or a sex pheromone, and this would be a metabolically economical method of producing different chemical messages at different times in the long adult lives of these beetles.

This phenomenon of altering a pheromone blend over time does not seem to occur with sex pheromones, since these normally act over short periods of time. However, in bark beetles, different pheromone components are released at various stages of the aggregation process (Birch, 1984), to elicit different behavioural responses, which could be considered as "blend changes", although they are not usually so described.

Further work on the pheromone production by individual males is required to determine if blend changes do occur. Similarly, further studies are needed on the behaviour of insects within aggregations, and within grain, rather than in bioassay arenas. Unfortunately, little information is available on the behaviour of insects in grain stores, nor of the composition or duration of aggregations, since they are destroyed immediately on discovery. Future work in this area is essential for a fuller understanding of the complex responses of the saw-toothed grain beetle to pheromones and food odours.

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